

PATENT COOPERATION TREATY

PCT

NOTIFICATION OF THE RECORDING
OF A CHANGE(PCT Rule 92bis.1 and
Administrative Instructions, Section 422)

Date of mailing (day/month/year) 27 April 1998 (27.04.98)	From the INTERNATIONAL BUREAU To: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria Arnold & Siedsma Sweelinckplein 1 NL-2517 GK The Hague PAYS-BAS
Applicant's or agent's file reference L/SA49/89p	IMPORTANT NOTIFICATION
International application No. PCT/EP97/04759	International filing date (day/month/year) 22 August 1997 (22.08.97)

1. The following indications appeared on record concerning: <input checked="" type="checkbox"/> the applicant <input type="checkbox"/> the inventor <input type="checkbox"/> the agent <input type="checkbox"/> the common representative				
Name and Address K.U. LEUVEN RESEARCH & DEVELOPMENT Groot Begijnhof Benedenstraat 59 B-3000 Leuven Belgium	State of Nationality BE		State of Residence BE	
	Telephone No.			
	Facsimile No.			
	Teleprinter No.			
2. The International Bureau hereby notifies the applicant that the following change has been recorded concerning: <input checked="" type="checkbox"/> the person <input type="checkbox"/> the name <input checked="" type="checkbox"/> the address <input checked="" type="checkbox"/> the nationality <input checked="" type="checkbox"/> the residence				
Name and Address VLAAMS INTERUNIVERSITAIR INSTITUUT VOOR BIOTECHNOLOGIE Rijvisschestraat 118 B-9052 Zwijnaarde Belgium	State of Nationality BE		State of Residence BE	
	Telephone No.			
	Facsimile No.			
	Teleprinter No.			
3. Further observations, if necessary:				
4. A copy of this notification has been sent to: <input checked="" type="checkbox"/> the receiving Office <input type="checkbox"/> the designated Offices concerned <input type="checkbox"/> the International Searching Authority <input checked="" type="checkbox"/> the elected Offices concerned <input checked="" type="checkbox"/> the International Preliminary Examining Authority <input type="checkbox"/> other:				

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer B. Fitzgerald Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

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NOTIFICATION OF ELECTION
(PCT Rule 61.2)

To:

United States Patent and Trademark
Office
(Box PCT)
Crystal Plaza 2
Washington, DC 20231
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 21 April 1998 (21.04.98)	To: United States Patent and Trademark Office (Box PCT) Crystal Plaza 2 Washington, DC 20231 ETATS-UNIS D'AMERIQUE in its capacity as elected Office
International application No. PCT/EP97/04759	Applicant's or agent's file reference L/SA49/89p
International filing date (day/month/year) 22 August 1997 (22.08.97)	Priority date (day/month/year) 22 August 1996 (22.08.96)
Applicant VAN DE VEN, Willem, Jan, Marie et al	

1. The designated Office is hereby notified of its election made:

in the demand filed with the International Preliminary Examining Authority on:
20 March 1998 (20.03.98)

in a notice effecting later election filed with the International Bureau on:

2. The election was
 was not

made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland Facsimile No.: (41-22) 740.14.35	Authorized officer A. Karkachi Telephone No.: (41-22) 338.83.38
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PATENT COOPERATION TREATY

REC'D 30 OCT 1998
WIPO PCT

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INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference L/SA49/89p	FOR FURTHER ACTION		See Notification of Transmittal of International Preliminary Examination Report (PCT/IPEA/416)
International application No. PCT/EP97/04759	International filing date (day/month/year) 22/08/1997	Priority date (day/month/year) 22/08/1996	
International Patent Classification (IPC) or national classification and IPC C07K14/47			
Applicant VLAAMS INTERUNIVERSITAIR INSTITUUT...et al.			

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 7 sheets, including this cover sheet.

This report is also accompanied by ANNEXES, i.e., sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

These annexes consist of a total of sheets.

3. This report contains indications relating to the following items:

- I Basis of the report
- II Priority
- III Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
- IV Lack of unity of invention
- V Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement
- VI Certain documents cited
- VII Certain defects in the international application
- VIII Certain observations on the international application

Date of submission of the demand 20/03/1998	Date of completion of this report 28.10.98
Name and mailing address of the IPEA/  European Patent Office D-80298 Munich Tel. (+49-89) 2399-0, Tx. 523656 epmu d Fax: (+49-89) 2399-4465	Authorized officer SCHEFFZYK, I Telephone No. (+49-89) 2399-8602



**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP97/04759

I. Basis of the report

1. This report has been drawn on the basis of (substitute sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to the report since they do not contain amendments.):

Description, pages:

1-73 as originally filed

Claims, No.:

1-25 as originally filed

Drawings, sheets:

1/18-18/18 as originally filed

2. The amendments have resulted in the cancellation of:

the description, pages:
 the claims, Nos.:
 the drawings, sheets:

3. This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

4. Additional observations, if necessary:

IV. Lack of unity of invention

1. In response to the invitation to restrict or pay additional fees the applicant has:

restricted the claims.
 paid additional fees.
 paid additional fees under protest.
 neither restricted nor paid additional fees.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP97/04759

2. This Authority found that the requirement of unity of invention is not complied and chose, according to Rule 68.1, not to invite the applicant to restrict or pay additional fees.
3. This Authority considers that the requirement of unity of invention in accordance with Rules 13.1, 13.2 and 13.3 is
 - complied with.
 - not complied with for the following reasons:
see separate sheet
4. Consequently, the following parts of the international application were the subject of international preliminary examination in establishing this report:
 - all parts.
 - the parts relating to claims Nos. .

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes: Claims
	No: Claims 1-25
Inventive step (IS)	Yes: Claims
	No: Claims 1-25
Industrial applicability (IA)	Yes: Claims 1-25
	No: Claims

2. Citations and explanations

see separate sheet

VI. Certain documents cited

1. Certain published documents (Rule 70.10)

and / or

2. Non-written disclosures (Rule 70.9)

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT**

International application No. PCT/EP97/04759

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:

see separate sheet

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP97/04759

SECTION IV -----

The common inventive concept linking together the subject-matter of the present claims can be seen in the provision of genes involved in tumorigenesis. However, taking into account that hitherto a number of such genes already have been isolated in the art (see e.g. Weinberg R.A. et al., *Science*, vol. 254, no. 5035, 22.11.91, pp. 1138-1146 (1)) and considering further that no other characteristic technical features common to the claimed genes can be distinguished (the PLAG and the CTNNB1 genes are structurally unrelated and independently from each other and are involved in different tumours) the IPEA is of the opinion that this common concept no longer exists. Correspondingly, present claims relate not to one but to three separate inventions not linked by a common inventive concept, namely:

**invention 1: isolated gene implicated in tumorigenesis having
the nucleotide sequence of any one of the members of the PLAG subfamily of zinc
finger protein genes and the use thereof (claim 2-4 and claims 1 and 6-11, 13, 16-
19, 21, partially;**

**invention 2: isolated gene implicated in tumorigenesis having
the nucleotide sequence of the CTNNB1 gene and the use thereof (claim 5 and
claims 1 and 6-11, 13, 16-19, 21, partially and**

**invention 3: Method for isolating other tumorigenesis genes
(T-genes) (claims 12, 14, 15, 20, 22, 23, 24 and 25 and claim 16, partially).**

Accordingly, present application does not comply with the requirements of Rule 13.1-13.3 PCT.

SECTION V -----

- 1). In so far as the modified versions of the nucleotide sequence encoding any one of the members of the PLAG subfamily of zinc finger proteins or CTNNB1 protein are

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP97/04759

not precisely defined in the claims the subject-matter of present claims 1-10 is not clearly delimitated from any hitherto known genes involved in tumorigenesis.

Concerning claim 5 which is directed to the CTNNB1 gene it is noted that even if said claim was clearly limited to the CTNNB1 gene said claim would not be novel either since said gene already has been cloned and sequenced in the prior art (see e.g. Nollet F. et al., Genomics 32, pp. 413-424 (1996) (2).

Furthermore, in so far as the subject-matter of claims 1-8 is not clearly delimitated from hitherto readily available genes implicated in tumorigenesis at present the subject-matter of claims 11-25 cannot be considered to be novel either.

As regards claims 6-10 it is pointed out that an indication of use in a compound claim has no limiting effect, i.e. the scope of such a claim is directed to the compound *per se*.

Thus, at present claims 1-25 do not comply with the requirements of Art. 33(2) PCT.

- 2). Moreover, considering that both the PLAG gene family and the CTNNB1 gene are known *per se* in the art and considering further that it is also known in the art that these genes are involved in tumorigenesis the presence of an inventive step for the subject-matter of present claims cannot be acknowledged either.
Thus, claims 1-25 do not meet the requirements of Art 33(3) PCT.

SECTION VI -----

Genes, Chromosomes and Cancer, vol. 17, no. 3, November 1996, pp. 166-171,
E. Roijer et al.

EMBL Database, Heidelberg, DE, Acc.no. U72621, 22.10. 96, A. Abdollahi et al.

**INTERNATIONAL PRELIMINARY
EXAMINATION REPORT - SEPARATE SHEET**

International application No. PCT/EP97/04759

Oncogene, vol. 14, no. 16, 24.04.97, pp. 1973-1979, A. Abdollahi et al.

SECTION VIII-----

- 1). The terms "modified versions", "elongated versions", "fragments thereof", "practically usable fragments thereof" and "T-gene related macromolecule" written in the claims are vague and thus render the scope of the claims unclear (Art. 6 PCT).
- 2). As regards claim 13, feature d) it is noted that the expression PLAG has no general meaning in the art but is only an arbitrary designation and thus also renders the scope of said claim unclear.
- 3). Claim 14, feature d) is obscure :"...a part of the protein encoded by the remaining part of the T-gene(?) and against a part of the protein encoded by the substitution part of the T-gene (?)".

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference L/SA49/89p	FOR FURTHER ACTION see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.	
International application No. PCT/EP 97/04759	International filing date (day/month/year) 22/08/1997	(Earliest) Priority Date (day/month/year) 22/08/1996
Applicant K.U. LEUVEN RESEARCH & DEVELOPMENT et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 6 sheets.

It is also accompanied by a copy of each prior art document cited in this report.

1. Certain claims were found unsearchable (see Box I).
2. Unity of invention is lacking (see Box II).
3. The international application contains disclosure of a nucleotide and/or amino acid sequence listing and the international search was carried out on the basis of the sequence listing
 - filed with the international application.
 - furnished by the applicant separately from the international application,
 - but not accompanied by a statement to the effect that it did not include matter going beyond the disclosure in the international application as filed.
 - Transcribed by this Authority
4. With regard to the title, the text is approved as submitted by the applicant.
 - the text has been established by this Authority to read as follows:
5. With regard to the abstract,
 - the text is approved as submitted by the applicant.
 - the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this International Search Report, submit comments to this Authority.
6. The figure of the drawings to be published with the abstract is:

Figure No. _____

 - as suggested by the applicant.
 - because the applicant failed to suggest a figure.
 - because this figure better characterizes the invention.

None of the figures.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/04759

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:
2. Claims Nos.: 6-23 partially because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
claims 6-23 could be searched only incompletely because they lack technical disclosure (Art. 6 PCT and PCT Search Guidelines, Chapter III, 3.7)
3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

B x II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.
2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:
4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims : 1 (partially), 2-4 (completely) and 6-25 (partially)

Isolated gene implicated in tumorigenesis, having the nucleotide sequence of any one of the members of the PLAG subfamily of zinc finger proteins

2. Claims : 1 (partially), 5 (completely) and 6-25 (partially)

Isolated gene implicated in tumorigenesis, having the nucleotide sequence of the CTNNB1 gene.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP 97/04759A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C07K14/47 C12Q1/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C07K C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	N.K. SPURR ET AL.: "Report of the second international workshop on human chromosome 8 mapping 1994" CYTOGENETICS AND CELL GENETICS, vol. 68, no. 3-4, 1995, BASEL CH, pages 148-155, XP002036781 * the whole document, esp. p153, column 2 * ---	1-4
A	WO 89 09777 A (ARCH DEVELOPMENT CORPORATION) 19 October 1989 see the whole document ---	1
A	DE 44 35 919 C (DEUTSCHES KREBSFORSCHUNGZENTRUM) 7 December 1995 see the whole document ---	1
		-/-

 Further documents are listed in the continuation of box C. Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

T later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

X document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

Y document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

& document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 June 1998	13-07- 1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer De Kok, A

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/04759

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>A. ABDOLLAHI ET AL.: "Identification of a gene which shows decreased expression in malignantly transformed rat ovarian surface epithelial cells" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 37, no. 0, 20 April 1996, WASHINGTON US, page 242 XP002036823 * abstract nr. 1654</p> <p>---</p>	1,4
P,X	<p>E. ROIJER ET AL.: "Identification of a yeast artificial chromosome spanning the 8q12 translocation breakpoint in pleiomorphic adenomas with t(3;8)(p21;q12)" GENES CHROMOSOMES AND CANCER, vol. 17, no. 3, November 1996, NEW YORK US, pages 166-171, XP002036782 cited in the application see the whole document</p> <p>---</p>	1-4,6-23
P,X	<p>EMBL DATABASE, Heidelberg, DE, Acc.nr. U72621, 22 October 1996, A. Abdollahi et al., "Identification of a gene containing zinc-finger motifs based on lost expression in malignantly transformed rat ovarian surface epithelial cells". XP002036784 see abstract</p> <p>---</p>	1,4
P,X	<p>A. ABDOLLAHI ET AL.: "Identification of a zinc-finger gene at 6q25: a chromosomal region implicated in development of many solid tumors" ONCOGENE, vol. 14, no. 16, 24 April 1997, OXFORD GB, pages 1973-1979, XP002036783 see the whole document</p> <p>---</p>	1,4
X	<p>F NOLLET ET AL: "Genomic organization of the human beta-catenin gene (CTNNB1)" GENOMICS, vol. 32, no. 3, March 1996, NEW YORK US, pages 413-424, XP002047790 cited in the application see the whole document</p> <p>---</p> <p>-/-</p>	1,5-25

INTERNATIONAL SEARCH REPORT

International Application No
PCT/EP 97/04759

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. HUELSEN ET AL.: "E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton" JOURNAL OF CELL BIOLOGY, vol. 127, 1994, NEW YORK US, pages 2061-2069, XP002047791 see the whole document ---	1,5-25
X	C KRAUS ET AL.: "Localization of the human beta-catenin gene (CTNNB1) to 3p21: A region implicated in tumor development" GENOMICS, vol. 23, no. 1, 1994, NEW YORK US, pages 272-274, XP002047792 cited in the application see the whole document ---	1,5-25
X	J. VAN HENGEL ET AL.: "Assignment of the human beta-catenin gene (CTNNB1) to 3p22-p21.3 by fluorescence in situ hybridization" CYTOGENETICS AND CELL GENETICS, vol. 70, no. 1-2, 1995, BASEL CH, pages 68-70, XP002047793 see the whole document ---	1,5-25
A	RABBITS T H: "CHROMOSOMAL TRANSLOCATIONS IN HUMAN CANCER" NATURE., vol. 372, 10 November 1994, LONDON GB, pages 143-149, XP002056672 see the whole document ---	1
A	WEINBERG R A: "TUMOR SUPPRESSOR GENES" SCIENCE., vol. 254, no. 5035, 22 November 1991, LANCASTER, PA US, pages 1138-1146, XP002056673 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/EP 97/04759

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO 8909777 A	19-10-1989	US	5206152 A	27-04-1993
DE 4435919 C	07-12-1995	WO EP	9611267 A 0784680 A	18-04-1996 23-07-1997



INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C07K 14/47, C12Q 1/68		A2	(11) International Publication Number: WO 98/07748 (43) International Publication Date: 26 February 1998 (26.02.98)
(21) International Application Number: PCT/EP97/04759 (22) International Filing Date: 22 August 1997 (22.08.97)		(74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).	
<p>(30) Priority Data: 96202339.6 22 August 1996 (22.08.96) EP (34) Countries for which the regional or international application was filed: AT et al.</p> <p>97200130.9 17 January 1997 (17.01.97) EP (34) Countries for which the regional or international application was filed: AT et al.</p>		<p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p>	
<p>(71) Applicants (for all designated States except US): K.U. LEUVEN RESEARCH & DEVELOPMENT [BE/BE]; Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE). HOLDINGBOLAGET VID GÖTEBORGS UNIVERSITET AB [SE/SE]; Medicinaregatan 8, S-413 90 Göteborg (SE).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): VAN DE VEN, Willem, Jan, Marie [NL/BE]; Lei 8A bus 42, B-3000 Leuven (BE). STENMAN, Karl, Göran, David [SE/SE]; Fältskärsgatan 7, S-426 76 Västra Frölunda (SE). KAS, Koen, Pieter [BE/BE]; Kasteelrei 115, B-2110 Wijnegem (BE). VOZ, Marianne, Léontine [BE/BE]; Rue Marcel Hicter 14, B-4300 Waremme (BE).</p>		<p>Published Without international search report and to be republished upon receipt of that report.</p>	

(54) Title: PLAG GENE FAMILY AND TUMORIGENESIS

(57) Abstract

The present invention relates to a novel gene family identified as T-genes and being implicated in tumorigenesis having the nucleotide sequence of any one of the strands of any one of the members of the PLAG and CTNNB1 gene families. The genes and their derivatives may be used in various diagnostic and therapeutic applications.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
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CM	Cameroon	KR	Republic of Korea	PT	Portugal		
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CU	Cuba	LC	Saint Lucia	RU	Russian Federation		
CZ	Czech Republic	LI	Liechtenstein	SD	Sudan		
DE	Germany	LK	Sri Lanka	SE	Sweden		
DK	Denmark	LR	Liberia	SG	Singapore		
EE	Estonia						

PLAG GENE FAMILY AND TUMORIGENESIS

The present invention relates to the identification of the PLAG gene family as a family of genes frequently associated with tumorigenesis. The invention in particular relates to the identification of one member of this gene family that is involved in benign tumors such as those with chromosome anomalies involving a particular region of the long arm of chromosome 8, for instance but not restricted to pleomorphic adenomas of the salivary glands with involvement of chromosome 8q12 and a translocation partner chromosome, very frequently chromosome 3 [i.e. t(3;8)(p21;q12)]. In addition, the invention relates to another member of this novel family as the gene whose expression is frequently abrogated in malignant tumors such as for example but not restricted to malignant salivary gland tumors. Furthermore, the invention concerns the identification of the CTNNB1 gene as a prototype of tumor-specific breakpoint region genes and frequent fusion partner of the PLAG genes. The invention relates in particular to the use of the members of the PLAG gene family and corresponding fusion partner genes as well as their derivatives in diagnosis and therapy.

Multiple independent cytogenetic studies have firmly implicated chromosome region 8q11-13 in the following tumor types: pleomorphic adenomas of the salivary glands (high frequency), pleomorphic adenomas of the lacrimal glands (high frequency), lipoblastomas (high frequency), solitary lipomas (low frequency), rhabdomyosarcomas (low frequency), and renal cell carcinomas (low frequency). As an illustration, we here focus on pleomorphic adenomas of the salivary gland. This type of tumor constitutes a benign epithelial tumor that originates from the major and minor salivary glands. It is the most common type of salivary gland tumor and accounts for almost 50% of all neoplasms in these organs; 85% of the tumors are found in the parotid gland, 10% in the minor salivary glands and 5% in the submandibular gland. About 50% of these adenomas appear to have a normal karyotype but cytogenetic studies have also

revealed recurrent specific chromosome anomalies. Frequently observed anomalies include aberrations of chromosome 8, usually involving the 8q12 region with as most common aberration a $t(3;8)(p21;q12)$, and aberrations of chromosome 12, usually translocations involving region 12q13-q15. Non-recurrent clonal chromosome abnormalities have also been reported. The highly specific pattern of chromosome rearrangements with consistent breakpoints at 8q12 and 12q13-q15 suggests that these chromosomal regions harbour genes that might be implicated in the development of these tumors.

In previous physical mapping studies, the chromosome 8q12 breakpoint in pleomorphic adenoma of the salivary glands was mapped between MOS and PENK. In an effort to molecularly clone the gene affected by the chromosome 8q12 aberrations in the various tumors, the present inventors chose directional chromosome walking as a structural approach to define the DNA region encompassing these breakpoints.

As a starting point for chromosome walking, the MOS locus was used. During these walking studies, YAC clones corresponding to eight different loci in 8q11-12 were isolated and mapped by FISH analysis. Initially, the breakpoints were mapped within a 1 Mb region flanked by MOS proximally and by the genetic marker D8S166 distally. One YAC (CEPH 166F4) within this region was shown to span the $t(3;8)$ breakpoint in two tumors. Subsequently, it was shown by FISH that the chromosomal breakpoints as present in a number of primary pleomorphic adenomas were clustered within a small chromosomal segment which has been designated pleomorphic adenoma breakpoint cluster region (PA-BCR).

It has thus been found that essentially all breakpoints of chromosome 8q12 map in a region indicated herein as PA-BCR. Further research revealed that in this region a member of the PLAG gene family, the PLAG1 gene, can be identified as the breakpoint target gene and postulated tumor aberrant growth gene. The PLAG gene family is a subclass of the zinc finger gene superfamily. The CTNNB1 gene was identified as the fusion partner gene of PLAG1. Molecular analysis of the

chromosome breakpoints revealed that the chromosome translocations resulted in gene fusions. Fusions appeared to occur in the 5'-non-coding regions of both genes (PLAG1 and CTNNB1), exchanging regulatory control elements while preserving the coding sequences. Due to the t(3;8)(p21;q12), PLAG1 is activated and expression of CTNNB1 is down-regulated. Activation of PLAG1 was also observed in adenomas with variant translocations, i.e. t(5;8) and t(8;15). The results of the present inventors indicate that PLAG1 activation due to promoter swapping is a crucial event in salivary gland tumorigenesis.

The preferential fusion partner of PLAG1 is the CTNNB1 gene, which encodes β -catenin, a cytoplasmic protein of about 88 kD. Its frequent involvement in pleomorphic adenomas point towards a critical role of the CTNNB1 gene. The major role of CTNNB1 may be to provide an active promoter in front of the PLAG1 gene. However, since the observed promoter swapping between PLAG1 and CTNNB1 leads to down-regulation of CTNNB1 expression, it may also lead to other physiological consequences, especially since β -catenin is a protein that has been implicated in highly diverse processes, such as human colon cancer, epithelial cell adhesion, embryonal axis formation in Xenopus, and pattern formation in Drosophila.

According to the present invention the aberrations in the PLAG1 gene on chromosome 8 and the CTNNB1 gene on chromosome 3 have been used as a model to reveal the more general concept of the involvement of members of these gene families in tumorigenesis. Although both gene families are known per se, up till the present invention the correlation between these families and tumor inducing chromosome aberrations, like translocations, deletions, insertions and inversions, has not been anticipated. Furthermore, until now, it was not previously demonstrated that alterations in the physiological expression level of the members of the gene family are probably also implicated in tumor development. According to the invention, it was demonstrated that in normal adult cells the expression level of the PLAG1 gene is

practically undetectable, whereas in aberrantly growing cells, the expression level is significantly increased. It appears that PLAG1 is a oncogene.

The research that led to the present invention 5 indicates that it is probable that abberations in the PLAG1 gene, either in its sequence and/or its expression, will usually lead to a benign tumor. Furthermore, preliminary research indicated that expression of PLAG1 is increased in various types of malignant salivary gland tumors.

10 Another member of the PLAG gene family is the PLAG2 gene. The present inventors have identified this gene, determined its nucleotide sequence and predicted its amino acid sequence. It was found that PLAG2 mapped to a region frequently deleted in malignant salivary gland tumors 15 (6q24). Thus, contrary to PLAG1, PLAG2 may be a tumorsuppressor gene.

The present invention now provides for a tool to investigate these theories and may ultimately lead to a means for distinguishing between benign and malignant 20 tumors. This knowledge can then lead to more efficient methods of treatment. Thus, the present invention now provides for the members of the gene families or derivatives thereof in isolated form and their use in diagnostic and therapeutic applications. Furthermore, the knowledge on the 25 location and nucleotide sequence of the genes may be used to study their rearrangements or expression and to identify a possible increase or decrease in their expression level and the effects thereof on cell growth. Based on this information diagnostic tests or therapeutic treatments may be designed. 30

In this application, "PLAG" will be used to indicate the involvement of these types of genes in various types of tumors, not necessarily restricted to pleomorphic adenomas of the salivary glands. The term refers to all members of 35 the PLAG gene family involved in non-physiological proliferative growth, and in particular involved in benign or malignant tumors. Members of the PLAG gene family show homology between zinc finger domains that are typical for

the PLAG1 gene. However, according to the invention it was furthermore found that even breaks outside the actual genes but in the vicinity thereof might result in aberrant growth. The term "PLAG gene" is therefore also intended to include 5 the immediate vicinity of the gene. The skilled person will understand that the "immediate vicinity" should be understood to include the surroundings of the gene in which the breaks or mutations will result in the above-defined non-physiological proliferative growth.

10 The term "Tumorigenesis gene" or "T-gene" will be used to indicate all members of this novel PLAG gene family and their corresponding translocation or fusion partners, like CTNNB1.

15 The term "wildtype cell" is used to indicate the cell not harbouring an aberrant chromosome. "Wildtype" or "normal" chromosome refers to a non-aberrant chromosome.

20 The present invention provides for various diagnostic and therapeutic applications that are based on the information that may be derived from the genes. This information not only encompasses its nucleotide sequence or the amino acid sequence of the gene product derived from the gene, but also involves the levels of transcription or translation of the gene.

25 The invention is thus two-fold. On the one hand the aberration in cell growth may be directly or indirectly caused by the physical breaks that occur in the gene or its vicinity. On the other hand the aberration in cell growth may be caused by a non-physiological expression level of the gene. This non-physiological expression level may be caused 30 by the break, or may be due to another stimulus that activates or deactivate the gene. At present, the exact mechanism or origin of the aberrant cell growth is not yet completely unraveled. However, exact knowledge at this time is not necessary to define methods of diagnosis or treatment.

35 Diagnostic methods according to the invention are thus based on the fact that an aberration in a chromosome results in a detectable alteration in the chromosomes' appearance or biochemical behaviour. A translocation, for example will

result in a first part of the chromosome (and consequently of a PLAG gene) having been substituted for another (second) part (further referred to as "first and second substitution parts"). The first part will often appear someplace else on 5 another chromosome from which the second part originates. As a consequence hybrids will be formed between the remaining parts of both (or in cases of triple translocations, even more) chromosomes and the substitution parts provided by their translocation partners. Since it has now been found 10 that the breaks occur in a PLAG gene, this will result in hybrid gene products of that PLAG gene. Markers, such as hybridising molecules like RNA, DNA or DNA/RNA hybrids, or antibodies will be able to detect such hybrids, both on the DNA level, and on the RNA or protein level.

15 For example, the transcript of a hybrid will still comprise the region provided by the remaining part of the gene/chromosome but will miss the region provided by the substitution part that has been translocated. In the case of inversions, deletions and insertions the gene may be equally 20 afflicted.

Translocations are usually also cytogenetically detectable. The other aberrations are more difficult to find because they are often not visible on a cytogenetical level. The invention now provides possibilities for diagnosing all 25 these types of chromosomal aberrations.

In translocations markers or probes based on the PLAG gene for the remaining and substitution parts of a chromosome in situ detect the remaining part on the original chromosome but the substitution part on another, the translocation 30 partner.

In the case of inversions for example, two probes will hybridise at a specific distance in the wildtype gene. This distance might however change due to an inversion. In situ such inversion may thus be visualized by labeling a set of 35 suitable probes with the same or different detectable markers, such as fluorescent labels. Deletions and insertions may be detected in a similar manner.

According to the invention the above in situ applications can very advantageously be performed by using FISH techniques. The markers are e.g. two cosmids one of which comprises exon 1 and the upstream region of the PLAG gene, 5 while the other comprises the last exon and its downstream region. Both cosmids are labeled with different fluorescent markers, e.g. blue and yellow. The normal chromosome will show a combination of both labels, thus giving a green signal, while the translocation is visible as a blue signal 10 on the remaining part of one chromosome (e.g. 8) while the yellow signal is found on another chromosome comprising the substitution part. In case the same labels are used for both probes, the intensity of the signal on the normal chromosome will be 100%, while the signal on the aberrant chromosomes 15 is 50%. In the case of inversions one of the signals shifts from one place on the normal chromosome to another on the aberrant one.

In the above applications a reference must be included for comparison. Usually only one of the two chromosomes is 20 afflicted. It will thus be very convenient to use the normal chromosome as an internal reference. Furthermore it is important to select one of the markers on the remaining or unchanging part of the chromosome and the other on the substitution or inverted part. In the case of the PLAG1 gene 25 of chromosome 8, breaks are usually found in the 5'-UTR as is shown by the present invention. Probes based on the 5'- and 3'-UTR are thus very useful. As an alternative a combination of probes based on both translocation or fusion partners may be used, e.g. 5'-UTR of PLAG1 and 3'-UTR of 30 CTNNB1.

"Probes" as used herein should be widely interpreted and include but are not limited to linear DNA or RNA strands, Yeast Artificial Chromosomes (YACs), or circular DNA forms, such as plasmids, phages, cosmids etc..

35 These in situ methods may be used on metaphase and interphase chromosomes.

Besides the above described in situ methods various diagnostic techniques may be performed on a more biochemical

level, for example based on alterations in the DNA, RNA or protein or on changes in the physiological expression level of the gene.

Basis for the methods that are based on alterations in the chromosome's biochemical behavior is the fact that by choosing suitable probes, variations in the length or composition in the gene, transcript or protein may be detected on a gel or blot. Variations in length are visible because the normal gene, transcript(s) or protein(s) will appear in another place on the gel or blot than the aberrant one(s). In case of a translocation more than the normal number of spots will appear.

Based on the above principles the invention thus relates to a method of diagnosing cells having a non-physiological proliferative capacity, comprising the steps of taking a biopsy of the cells to be diagnosed, isolating a suitable PLAG gene-related macromolecule therefrom, and analysing the macromolecule thus obtained by comparison with a reference molecule originating from cells not showing a non-physiological proliferative capacity, preferably from the same individual. The PLAG gene-related macromolecule may thus be a DNA, an RNA or a protein. The PLAG gene may be either a member of the PLAG1 family or of the translocation partner gene family of which the CTNNB1 gene is the prototype.

In a specific embodiment the diagnostic method of the invention comprises the steps of taking a biopsy of the cells to be diagnosed, extracting total RNA thereof, preparing a first strand cDNA of the mRNA species in the total RNA extract or poly-A-selected fraction(s) thereof, which cDNA comprises a suitable tail; performing a PCR using a PLAG gene-specific primer and a tail-specific primer in order to amplify PLAG gene-specific cDNA's; separating the PCR products on a gel to obtain a pattern of bands; evaluating the presence of aberrant bands by comparison to wildtype bands, preferably originating from the same individual.

As an alternative amplification may be performed by means of the Nucleic Acid Sequence-Based Amplification (NASBA) technique [Compton, J. (1991) Nucleic acid sequence-

based amplification. *Nature* 350, 91-92] or variations thereof.

In another embodiment the method comprises the steps of taking a biopsy of the tumor to obtain cells to be diagnosed, isolating total protein therefrom, separating the total protein on a gel to obtain essentially individual bands, optionally transferring the bands to a Western blot, hybridising the bands thus obtained with antibodies directed against a part of the protein encoded by the remaining part 10 of the PLAG gene and against a part of the protein encoded by the substitution part of the PLAG gene; visualising the antigen-antibody reactions and establishing the presence of aberrant bands by comparison with bands from wildtype proteins, preferably originating from the same individual.

15 In a further embodiment the method comprises taking a biopsy of the tumor to obtain cells to be diagnosed; isolating total DNA therefrom; digesting the DNA with one or more so-called "rare cutter" (typically "6- or more cutters") restriction enzymes; separating the digest thus 20 prepared on a gel to obtain a separation pattern; optionally transferring the separation pattern to a Southern blot; hybridising the separation pattern in the gel or on the blot with a set of probes under hybridising conditions; visualising the hybridisations and establishing the presence 25 of aberrant bands by comparison to wildtype bands, preferably originating from the same individual.

Changes in the expression level of the gene may be detected by measuring mRNA levels or protein levels by means of a suitable probe.

30 Diagnostic methods based on abnormal expression levels of the gene may comprise the steps of taking a sample of the tumor to obtain cells to be diagnosed; isolating mRNA therefrom; and establishing the presence and/or the (relative) quantity of mRNA transcribed from the PLAG gene 35 of interest in comparison to a control. Establishing the presence or (relative) quantity of the mRNA may be achieved by amplifying at least part of the mRNA of the PLAG gene by means of RT-PCR or similar amplification techniques. In an

alternative embodiment the expression level may be established by determination of the presence or the amount of the gene product (e.g. protein) by means of for example monoclonal antibodies.

5 The diagnostic methods of the invention may be used for diseases wherein cells having a non-physiological proliferative capacity are selected from the group consisting of benign tumors, such as the tumors pleomorphic adenomas of the salivary glands, lipoblastomas, uterine leiomyomas, and 10 other benign tumors as well as various malignant tumors, including but not limited to sarcomas (e.g. rhabdomyosarcoma) and leukemias and lymphomas.

As already indicated above, it has been found that in certain malignant tumors the expression level of the PLAG 15 genes is increased. Another aspect of the invention thus relates to the implementation of the identification of the PLAG genes in therapy. The invention for example provides anti-sense molecules or expression inhibitors of the PLAG gene for use in the treatment of diseases involving cells 20 having a non-physiological proliferative capacity by modulating the expression of the gene.

The invention thus provides derivatives of the PLAG gene and/or its immediate environment for use in diagnosis and the preparation of therapeutical compositions, wherein 25 the derivatives are selected from the group consisting of sense and anti-sense cDNA or fragments thereof, transcripts of the gene or fragments thereof, antisense RNA, triple helix inducing molecule or other types of "transcription clamps", fragments of the gene or its complementary strand, 30 proteins encoded by the gene or fragments thereof, protein nucleic acids (PNA), antibodies directed to the gene, the cDNA, the transcript, the protein or the fragments thereof, as well as antibody fragments. Besides the use of direct derivatives of the genes and their surroundings (flanking 35 sequences) in diagnosis and therapy, other molecules, like expression inhibitors or expression enhancers, may be used for therapeutic treatment according to the invention. An

example of this type of molecules are ribozymes that destroy RNA molecules.

Bsides the above-described therapeutic and diagnostic methods, the principles of the invention may also be used 5 for producing a transgenic animal model for testing pharmaceuticals for treatment of PLAG-related malignant or benign tumors. One of the examples describes the production of such an animal model.

It is to be understood that the principles of the 10 present invention are described herein for illustration purposes only with reference to the PLAG1 gene mapping at chromosome 8q12 and the CTNNB1 gene on chromosome 3p21. Based on the information provided in this application the 15 skilled person will be able to isolate and sequence corresponding genes of the gene family and apply the principles of this invention by using the gene and its sequence without departing from the scope of the general concept of this invention.

The present invention will thus be further elucidated 20 by the following examples which are in no way intended to limit the scope thereof.

EXAMPLES

25 EXAMPLE 1

1. Introduction

This example describes the isolation and analysis of overlapping YAC clones and the establishment of a YAC contig (set of overlapping clones), which spans genomic DNA around 30 the MOS locus and includes the translocation breakpoints, $t(3;8)(p21;q12)$, of pleomorphic salivary glands (Fig. 8). Pleomorphic adenomas are benign epithelial tumors originating from the major and minor salivary glands. Cytogenetic analysis has indicated that these tumors mainly 35 display chromosome breakpoints in region q12 of chromosome 8. In previous studies we have shown that these breakpoints are located in the 9 cM interval between MOS / D8S285 and D8S260. Here we report directional chromosome walking stu-

dies starting from D8S260 and D8S285, resulting in, respectively, a rudimentary YAC contig of 5 Mb and a highly detailed YAC contig and long-range physical map of about 2 Mb. The latter YAC contig has at least double coverage and 5 consists of 34 overlapping YAC clones isolated from the CEPH and ICRF YAC libraries. Their insert sizes were estimated by contour-clamped homogeneous electric field (CHEF) gel electrophoresis. Chromosomal localization of the YACs was investigated by FISH analysis. On the basis of YAC insert and 10 insert-end derived DNA markers and (publicly known) sequence tag sites (STSS), with an average spacing of 65 kbp, as well as restriction enzyme analysis, a long-range physical map was established for this centromeric region of chromosome 8q12. Within the YAC contig, the relative positions of 15 various known genes and expressed sequence tag sites was determined. This YAC contig constitutes the basis for the construction of a transcriptional map of this region. FISH analysis using YACs and cosmids, corresponding to STSSs in this YAC contig, already revealed that all but two of the 20 chromosome 8q12 breakpoints in the primary tumors that were tested, mapped within a 300 kb interval between the MOS proto-oncogene and STS EM156.

2. Materials and methods

25 2.1. Pleomorphic adenomas of the salivary glands and cytogenetic analysis.

Primary pleomorphic adenomas of the salivary glands were obtained from patients at the time of surgery. Primary cultures and chromosome preparations were established and 30 analyzed as described before (Röijer et al., 1996). The primary tumors with their aberrations used in this study are listed in Table 1. Slides for FISH were prepared from cells stored in fixative at -20°C.

35 2.2. Fluorescence *in situ* hybridization (FISH)

Slides for FISH were aged for 2-10 days, or were treated with 2x SSC (pH 7.0) at 37°C for 30 min, and subsequently denatured in 70% formamide in 2x SSC (pH 7.0), at

75°C for 2-6 minutes. Cosmid and YAC DNAs (1 µg) were labelled with biotin-16-dUTP (Boehringer Mannheim) by nick translation. Labeled DNA was purified through a Sephadex G50 column (Pharmacia), coprecipitated with 50 mg sonicated 5 human placenta DNA (Sigma), and dissolved in hybridization solution (50% formamide, 2x SSC pH 7.0, 50 mM sodium phosphate pH 7.0, 10% dextran sulfate). Hybridization and probe detection were carried out as previously described (Röijer et al., 1996). The alpha-satellite probe D8Z22 was obtained 10 from Oncor. Slides were examined in a Zeiss Axiophot epifluorescence microscope using the appropriate filter combinations. Fluorescence signals were digitalized, enhanced and analyzed using the ProbeMaster FISH image analysis system (Perceptive Scientific Instruments, Houston, 15 Texas). Color prints were produced using a Kodak XL 7700 monochrome continuous printer.

2.3 DNA preparations.

Extraction of genomic DNA and the Southern analyses 20 were performed as described previously (Schoenmakers et al., 1994). DNA from YACs, cosmids, PCR products and oligonucleotides was labelled using a variety of techniques. For FISH, cosmid clones or inter-Alu PCR products of YACs were biotinylated with biotin-11-dUTP (Boehringer) by nick translation. YAC DNA (100 ng) was amplified by inter Alu PCR (P1: 25 CTGCACCTCCAGCCTGGG, P2: TCCCAAAGTGCTGGGATTACAG). After initial denaturation for 5 min at 94 °C, 30 amplification cycles were performed each consisting of denaturation for 1 min at 94 °C, annealing for 30 sec at 37 °C and extension for 6 min 30 at 72 °C, and with a final extension at 72 °C for 10 min. Amplified DNA was purified with QIAQuick PCR Purification kit (Qiagen). For filter hybridizations, probes were radio-labelled with alpha-³²P-dCTP using random hexamers (Feinberg and Vogelstein, 1984). In case of PCR-products 35 smaller than 200 bp in size, a similar protocol was applied, but specific oligonucleotides were used to prime labelling reactions. Oligonucleotides were labelled using gamma-³²P-ATP.

2.4 YAC library screening.

YAC clones in this paper were isolated from the CEPH mark 1 (Albertsen et al., 1990) and CEPH mark 3 (Chumakov et al., 1992) YAC library, made available to us by the Centre 5 d'Etude du Polymorphisme Humain (CEPH). YACs were isolated using a combination of PCR-based screening and colony hybridization analysis (Green and Olson, 1990). Contaminating *Candida parapsylosis*, which was sometimes encountered, was eradicated by adding terbinafin to the growth medium (final 10 concentration of 25 microgram/ml). The isolated YAC clones were characterized by STS-content mapping, contour-clamped homogeneous electric field (CHEF) electrophoresis (Chu et al., 1986), restriction mapping and hybridization and FISH analysis.

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2.5 Cosmid library screening.

Cosmid clones were isolated from an arrayed human chromosome 8-specific cosmid library (Wood et al., 1992) obtained from Los Alamos National Laboratory (LANL). 20 LANL-derived cosmid clones are indicated by their microtiter plate addresses. Cosmid DNA was extracted using standard techniques involving purification over Qiagen tips (Qiagen).

2.6 Pulsed-field gel electrophoresis and Southern blot 25 analysis.

Pulsed-field gel electrophoresis and Southern blot analysis were performed exactly as described by Schoenmakers et al. (1994). Agarose plugs containing high-molecular weight YAC DNA (equivalent to about 1 x 10⁸ yeast cells) 30 were twice equilibrated in approximately 25 ml TE buffer (pH 8.0) for 30 min at 50 °C followed by two similar rounds of equilibration at room temperature. Plugs were subsequently transferred to round-bottom 2 ml eppendorf tubes and equilibrated two times for 30 min in 500 microliter of the appropriate 35 1 x restriction-buffer at the appropriate restriction temperature.

Thereafter, DNA was digested in the plugs according to the suppliers (Boehringer) instructions for 4 h using 30

units of restriction endonuclease per digestion reaction. After digestion, plugs along with appropriate molecular weight markers were loaded onto a 1% agarose / 0.25 x TBE gel, sealed with LMP-agarose and size fractionated on a CHEF 5 apparatus (Biorad) for 18 h at 6.0 V/cm using a pulse angle of 120 degrees and constant pulse times varying from 10 sec (separation up to 300 kbp) to 20 sec (separation up to 500 kbp). In the case of large restriction fragments, additional runs were performed, aiming at the separation of fragments 10 with sizes above 500 kbp.

Electrophoresis was performed at 14 °C in 0.25 x TBE. As molecular weight markers, lambda ladders (Promega) and home-made plugs containing lambda DNA cut with restriction endonuclease HindIII were used. After electrophoresis, gels 15 were stained with ethidium bromide, photographed, and UV irradiated using a stratalinker (Stratagene) set at 120 mJ. DNA was subsequently blotted onto Hybond N+ membranes (Amersham) for 4-16 h using 0.4 N NaOH as transfer buffer. After blotting, the membranes were dried for 15 min at 80 °C, 20 briefly neutralised in 2 x SSPE, and prehybridised for at least 3 h at 42 °C in 50 ml of a solution consisting of 50% formamide, 5 x SSPE, 5 x Denhardts, 0.1% SDS and 200 microgram/ml heparin. Filters were subsequently hybridised for 16 h at 42 °C in 10 ml of a solution consisting of 50% 25 formamide, 5 x SSPE, 1 x Denhardts, 0.1% SDS, 100 microgram/ml heparin, 0.5% dextran sulphate and 2-3 x 10⁶ cpm/ml of labelled probe. Thereafter, membranes were first washed two times for 5 min in 2 x SSPE/0.1% SDS at room temperature, then for 30 min in 2 x SSPE/0.1% SDS at 42 °C 30 and, finally, in 0.1 x SSPE/0.1% SDS for 20 min at 65 °C. Kodak XAR-5 films were exposed at -80 °C for 3-16 h, depending on probe performance. Intensifying screens (Kyokko special 500) were used.

Agarose plugs containing high-molecular weight yeast + 35 YAC DNA (equivalent to 1 x 10⁹ cells ml⁻¹) were prepared as described before (Schoenmakers et al., 1994). Plugs were thoroughly dialysed against four changes of 25 ml T10E1 (pH 8.0) followed by two changes of 0.5 ml 1 x restriction

buffer before they were subjected to either pulsed-field restriction enzyme mapping or YAC-end rescue.

2.7 PCR analysis.

PCR amplification was carried out using a Pharmacia LKB-Gene ATAQ Controller (Pharmacia/LKB) or a Perkin Elmer 9600 (Perkin-Elmer Cetus) in final volumes of respectively 50 and 25 microliter containing 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 0.01% gelatine, 2 mM dNTPs, 20 pmole of each amplimer, 1.25 units of AmpliTaq (Perkin-Elmer Cetus), and 100 ng (for superpools) or 20 ng (for pools) of DNA. After initial denaturation for 5 min at 94 °C, 30 amplification cycles were performed each consisting of denaturation for 1 min at 94 °C, annealing for 1 min at the appropriate temperature (see Table 3), and extension for 1 min at 72 °C, and with a final extension at 72 °C for 10 min. Results were evaluated by analysis of 10 microliter of the reaction product on 2% agarose gels.

2.8 Generation of STSs from YAC inserts and insert ends.

YAC-end rescue was performed using a vectorette-PCR procedure in combination with direct solid phase DNA sequencing, as described before (Geurts et al., 1994). STSs specific for YAC inserts were generated from inter-Alu PCR products, isolated using published oligonucleotides TC65 or 517 (Nelson et al., 1989) to which Sall-tails were added to facilitate cloning. Figure 10 shows some of these STSs. After sequence analysis, primer pairs were developed using the OLIGO computer algorithm (Rychlik, 1989). They were tested on human genomic DNA, basically according to procedures described above.

2.9 Nucleotide sequence analysis and oligonucleotides.

Nucleotide sequences were determined according to the dideoxy chain termination method using the T7 polymerase sequencing kit of Pharmacia/LKB or the dsDNA Cycle Sequencing System (GIBCO/BRL). Sequencing results were analyzed using an A.L.F. DNA sequencer™ (Pharmacia Biotech) on

standard 30 cm, 6% Hydrolink[®], Long RangeTM gels (AT Biochem). Sequence analysis utilized Lasergene (DNASTAR) and BLAST and BEAUTY searches (NCBI; Altschul et al., 1990). All oligonucleotides were purchased from Pharmacia Biotech.

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3. Results

3.1 Isolation of YAC clones to start chromosome walking.

According to radiation hybrid mapping data, band 8q12 is flanked by markers D8S165, D8S285 proximally, and by 10 D8S260 distally (Sapru et al., 1994). The estimated genetic distance between these markers is 9 cM (Gyapay et al., 1994). Using primer sets for these microsatellite markers, we selected sets of corresponding YAC clones. These YACs were used for initiating the walk towards the 8q12 translocation 15 breakpoints, meanwhile generating a long-range contig based upon STS-content mapping of these. Hence we started to construct a 8q12 centromeric YAC contig (from D8S165 / D8S285) and a 8q12 telomeric YAC contig (from D8S260). By fluorescent in situ hybridisation (FISH) experiments, we 20 could soon show that all but two of the translocation breakpoints in primary adenomas mapped within a 1 cM interval within the centromeric YAC contig (Röijer et al., 1996). Hence, the YAC contig of the telomeric side of 8q12 was not characterized in that much detail. The two YAC contigs could 25 not be linked to each other.

3.2 Assembly of a YAC contig starting from D8S285

In previous studies (Röijer et al., 1996) we have described the isolation of two overlapping YACs containing 30 D8S285, namely 935E9 and 166F4. These YACs, together with a MOS containing YAC (900g10157), were used as a starting point for a chromosome walking project to define the 8q12 centromeric region, as mentioned in the introduction. Initially, chromosome walking was performed bidirectionally. This 35 allowed us to hook up our 8q12 centromeric contig to a chromosome 8q11 YAC contig. Both contigs have the marker D8S593 in common. In the subsequent PCR based screening of the human CEPH YAC libraries with D8S108 and D8S166, we

isolated MegaYAC 946B7, which together with MegaYAC 935E9, formed the backbone of our centromeric contig.

In the bidirectional and subsequent unidirectional chromosome walking steps, the following general procedures 5 were used. First, rescuing and sequencing the ends of YAC clones resulted in DNA markers characterizing their left and right sides (Table 2). Based on sequence data on the ends of the YAC insert, as well as on inter-Alu PCR products, primer sets were developed for specific amplification of DNA, 10 establishing STSs (Table 3). Their location to 8q12-qter was determined by CASH as well as by FISH after corresponding YAC and/or cosmid clones were isolated. With the sequentially selected and evaluated primer sets, screening of the YAC and cosmid libraries was performed to isolate the building blocks for contig assembly. Hence, we established a 15 8q12 centromeric YAC contig consisting of 34 overlapping YAC clones, covering approximately 2 Mb of DNA (Fig. 1). The 34 YACs are between 160 kb and 1660 kb long. This YAC contig appeared to encompass the chromosome 8q12 breakpoints of all 20 but two primary adenomas studied. YAC 166F4 for instance, was already shown to cross the translocation breakpoint in two adenomas with t(3;8)(p21;q12) (Röijer et al., 1996). Characteristics of the YACs that were used to build this 25 contig are given in Table 2.

The YAC contig was constructed in parallel with the screening data of Whitehead Institute / MIT Center for Genome Research available via http://www-genome.wi.mit.edu/cgi-bin/contig/lookup_contig (Contigs WC8.7 and WC-157).

30 3.3 Physical mapping of polymorphic markers, ESTs and genes
All markers and genes which were or became publicly

available during our work were STS content mapped on our contig and those found positive were subsequently sublocalized by (primer) hybridization on YAC Southern blots. The 35 markers that were found to reside within the centromeric contig presented here were D8S1069 (WI-536), AFMB055WG9, D8S125, D8S108, D8S1661 (WI-5459), D8S166, D8S96, D8S1816, D8S285, D8S1516 (WI-3862), D8S1828 and D8S165. Furthermore,

besides the MOS proto-oncogene (Testa et al., 1988), two other genes could be positioned in our centromeric YAC contig, namely the YES related proto-oncogene LYN (Corey and Shapiro, 1994) and the preproenkephalin gene PENK (Tomfohrde et al., 1992). Finally, our contig contains 2 expressed sequence tags, namely WI-7754 (D8S1930), corresponding to LYN, and IB2045, not resembling any currently known gene. The genes encoding the kappa opioid receptor (OPRK1), (Yasuda et al., 1994) and interleukin-7 (IL7) (Brunton and Lupton, 1990) are not contained in our YAC contig.

3.4 Restriction map and putative CpG islands: Construction of a rare-cutter physical map from the 2 Mb YAC contig in the 8q12 centromeric region

15 Southern blots of total yeast plus YAC DNA, digested to completion with rare-cutter enzymes BssHII, KpnI, MluI, NotI, PvuI, SalI and SfiI (see Materials and Methods) and separated on CHEF gels, were hybridized sequentially with (i) the STS used for the initial screening of the YAC in 20 question, (ii) pYAC4 right arm sequences, (iii) pYAC4 left arm sequences, and (iv) a human Alu-repeat probe (BLUR-8). The long-range restriction map that was obtained in this way was completed by probing with PCR-isolated STSs/YAC end probes. Restriction maps of individual YAC clones were 25 aligned, and a consensus restriction map was established. The region was searched for CpG islands on the basis of the colocalization of sites for these rare-cutter enzymes (Fig. 1).

30 3.5 FISH mapping of 8q12 breakpoints in pleomorphic adenomas

In previous studies we have mapped the t(3;8)(p21;q12) breakpoint within a 1 Mb region flanked by MOS proximally and by the genetic marker D8S166 distally. One YAC within 35 this region (166F4) was shown to span the t(3;8) breakpoint in two primary pleomorphic adenomas (Röijer et al. (1996)). We have now extended these previous observations by analysis of additional tumors including cases with different variant

translocations (Table 1). Six of eight primary adenomas with 8q12 abnormalities had breakpoints mapping within YAC 166F4 (Fig. 2A). Also tumors with other 8q12 rearrangements than the classical t(3;8) had breakpoints within the same YAC, 5 demonstrating that this is the major breakpoint cluster region in pleomorphic adenomas with 8q12 abnormalities.

To fine map this region we isolated cosmids from an arrayed human chromosome 8-specific cosmid library (obtained from Los Alamos National Laboratory; Wood et al. (1992)) 10 using markers contained within YAC 166F4. Cosmid clones containing MOS, EM156 and CH129 were isolated and mapped by FISH (Fig. 1). The breakpoints in all six cases were localized between cosmids CEM1 (MOS) and CEM23 (EM156) (Fig. 2B and C). In for example CG 666, CEM1 was transposed 15 to 8p due to a pericentric inversion of the dicentric marker chromosome (Fig. 2B), while CEM23 remained in its normal position at 8q12 (Fig. 2C). In adenoma CG 682, which has an ins(8;3)(q12;p21.3p14.1), CEM23 spanned the insertion breakpoint (Röijer et al. (1997)).

20 Two of the eight adenomas tested, CG 650 and CG 787, had breakpoints clearly proximal to MOS. To further map these breakpoints we isolated two additional sets of YACs corresponding to CH37, D8S593 and the recently identified XRCC7 gene (Blunt et al. (1995)), respectively. In CG 650 25 the breakpoint was found to reside between ICRF YAC 900g10157 and YAC 898G12 (Fig. 1), and in CG 787 the breakpoint was found to reside within the XRCC7 containing YAC 943G4 (Fig. 2D). In the latter case, which judged from cytogenetics had an ins(8;7)(q11-12;p21p14), FISH analysis 30 revealed signals on both the der(7) and the der(8) chromosomes, demonstrating that this is not a simple 8:7-insertion but a complex rearrangement with at least two breakpoints on 8q, one of which maps within YAC 943G4 and the other proximal to this YAC.

3.6 Assembly of a YAC contig between D8S260 and D8S507

Two MegaYACs corresponding to D8S260, were used to start the chromosome walk from the telomeric side of chromosome 8q12. The YAC insert-ends from these two clones (783H12

and 814A6) were isolated and used for STS content mapping. The most telomeric STS (CH31), is present in two YACs also containing D8S510, and hence links the 8q12 YAC contig to a 8q13 specific YAC contig (Koenig et al., 1995). When we 5 started our physical mapping effort, only one polymorphic marker, D8S507, was described in between D8S285 and D8S260 (Gyapay et al., 1994). Initially, we were unable to isolate YACs containing D8S507. Instead we used the corresponding amplimers to isolate a cosmid containing D8S507 (cosmid 10 CEM3). An insert-ends of this cosmid clone (EM73) was then used to isolate corresponding MegaYACs (872E1, 882D9 and 957C4). It is unclear why these YACs were initially not recognised upon the YAC library screenings. This might have been due to either a less efficient PCR of D8S507 than of 15 EM73, or to an underrepresentation of the D8S507 YAC-containing yeast cells in the primary pools of the specific library we screened. The availability of two new polymorphic markers, D8S1505 and D8S1515 (WI-6879) allowed to link the two separate MegaYAC contigs around D8S260 and D8S507. The 20 composite contig consists of 23 CEPH MegaYACs and covers approximately 5 Mb of genomic DNA (Fig. 3). The polymorphic markers that were tested and found to reside in the telomeric YAC contig are D8S1151 (WI-1155), D8S260, D8S1075 (WI-943), D8S1505, D8S1515 (WI-6879), D8S1723, D8S507 and 25 D8S1957 (WI-9507).

One gene, CYP7, encoding cholesterol 7 α -Hydroxylase (and previously assigned to 8q11-12 using both mouse-human somatic cell hybrids and FISH (Cohen et al., 1992)) was mapped in this contig using a primerset that we developed on 30 publicly available sequence data (see Table 3). The YAC contig was constructed in parallel with the screening data of Whitehead Institute / MIT Center for Genome Research available via http://www-genome.wi.mit.edu/cgi-bin/contig/lookup_contig (Contigs WC8.8 and WC-727).

35

4. Discussion

In this example the physical map of 2 Mb of the centromeric region of chromosome 8q12 composed of 34 YAC

clones is presented. It is tagged by 31 markers with an average interval of 65 kb and was validated by FISH mapping. The frequency of chimaerism in the set of YACs studied in detail (YAC end rescue and/or restriction enzyme mapping and/or FISH) was slightly lower (6/26 = 23%) than the 30-40% expected (Van Ommen, 1993). These data provide the most detailed available physical map of the 8q12 region. The map was constructed in parallel with the establishment of a low-resolution map of the human genome and with the screening data of the Whitehead Institute/MIT Center for Genome Research. It was compared with data available electronically via Internet. The order of the markers on the physical map is in agreement with the 1996 Genethon human genetic linkage map (Dib et al. (1996)). In total, 31 markers/genes were ordered on the contig as follows:

D8S1069-CH274-TC65.2-AFMB055WG9-CH277-CH34-D8S125--
D8S108-EM76-D8S1661-CH122-D8S96-D8S166-CH69-CH33-D8S1816-PE-
NK-CH280-CH129-EM156-END2-CH283-D8S285-MOS-D8S1516-LYN-D8S1-
828-IB2045-D8S165-CH37-W1086.

A restriction map with the enzymes BssHII, KpnI, MluI, NotI, SalI, SfiI, SwaI was generated, revealing at least 7 putative CpG islands. Three of these island correspond to previously known genes (PENK, MOS and LYN), while the others might represent the 5' end of unidentified genes so far. Our 2 Mb map integrates cytogenetic, genetic and physical data of chromosome band 8q12. Chromosome band 8q12 seems to be very gene-poor: so far only 4 genes were shown to reside in both contigs (CYP7, PENK, MOS and LYN). This is consistent with the view that Giemsa-positive bands are chromosome regions rather low in G+C content and poor in genes (Craig and Bickmore, 1993). Chromosome 8q12 is partially conserved in synteny with mouse chromosome 4 (Mock et al., 1996). Comparative gene mapping however did not reveal any mouse gene or marker not present on our physical map.

Our physical map contains at least one disease locus, i.e. the pleomorphic adenoma translocation breakpoint. FISH analyses using YACs and cosmids revealed that the majority

of breakpoints clustered within a 300 kb region in both adenomas with the recurrent t(3;8) and in those with different sporadic 8q12 translocations. A cosmid from this region was also shown to span the breakpoint in an adenoma 5 with an ins(8;3) (Röijer et al. (1997)). This region is therefore likely to harbor the pleomorphic adenoma gene. The breakpoint region contains one known gene (MOS), one polymorphic marker (D8S285), and three new STSs (EM156, END2 and CH283). BLAST searches of these STSs revealed that CH283 10 displayed sequence identity with a publicly available EST (expressed sequence tag).

Two of the eight tumors in the present series had breakpoints outside the 300 kb breakpoint region. By FISH we could demonstrate that the breakpoints in both cases were 15 clearly proximal to MOS. In one case the breakpoint was mapped between MOS and YAC 898G12, and in the other case it was found to reside within YAC 943G4. The latter YAC maps at least 2 Mb centromeric to MOS. Since the two breakpoints are clearly different and also separate from those located in 20 the 300 kb breakpoint region it is unlikely that they affect the same gene. However, we can of course not rule out the possibility that these cases have additional rearrangements that may have escaped detection with the DNA probes used, and that they in fact all have rearrangements of a common 25 gene in the breakpoint region.

Recently, we have obtained molecular evidence for an additional breakpoint region distal to our telomeric contig (Röijer et al. (in manuscript)). Based on FISH using YACs derived from 8q13-21 we have estimated that these 30 breakpoints are about 15 to 20 cM telomeric to the 8q12 breakpoint cluster region. Cytogenetically, these breakpoints are very difficult, or impossible to distinguish from the classical 8q12 breakpoints. This is also true for the breakpoints located centromeric to MOS. Collectively, 35 our findings thus suggest that there are several genes in the proximal part of 8q that are affected by chromosome rearrangements in pleomorphic adenomas.

In addition to pleomorphic adenomas there are also a few other types of solid tumor with structural rearrangements involving 8q11-13, namely lipoblastomas (Dal Cin et al. (1994); Sawyer et al. (1994)), rhabdomyosarcomas 5 (Mitelman (1994)) and renal cell carcinomas (Elfving (1996)). It should also be mentioned that pleomorphic adenomas of the lacrimal glands show recurrent rearrangements of 8q12 (Hryncak et al. (1994)), including a t(3;8)(p21;q12). Because of the well known histopathological 10 and clinical similarities between pleomorphic adenomas originating from the salivary and lacrimal glands it is likely that the 8q12 abnormalities in these tumor types affect the same gene. As for the other tumor types mentioned it remains to be shown whether the rearrangements in these 15 cases also affect this gene. Preliminary FISH analysis of lipoblastoma with an 8q12 translocation breakpoint have shown that the breakpoint, as in pleomorphic adenomas, is distal to MOS (unpublished observations). Unfortunately no markers telomeric to MOS could be tested. FISH studies are 20 now in progress to resolve these critical questions.

Finally, a second YAC contig was established between D8S260 and D8S507, consisting of 23 CEPH MegaYACs and covering approximately 5 Mb. Both contigs represent about 75% of human chromosomal band 8q12, which extends over approximately 25 9 Mb of DNA. They will provide molecular access to new, previously unidentified genes from this region and especially to those directly affected by the chromosome 8q12 aberrations in pleomorphic adenomas of the salivary gland.

30 6. Figure legends

Figure 1.

Physical contig and STS marker map in proximal human 8q12. The upper line indicates the chromosome, with the 35 telomere to the left and the centromere to the right. DNA markers and genes are ordered across the top of the figure. Genes are underlined and previously known markers are indicated in bold. YAC clones are represented as lines together

with their names and are described in Table 2. STS locations are indicated by dashed lines. The length of the solid line indicates its approximative size. The size of YAC 391H2 and 5 392A6 is too large to fit within the constraints of the map, suggesting chimaerism. In these cases, the lenght of the YAC corresponds only to the markers it contains. YAC end clones used as STS are represented by arrows. Below the contig the consensus restriction maps for BssHII (B), KpnI (K), MluI (M), NotI (N), PvuI (P), SalI (S) and SfiI (Sf) are indicated. Verical arrows indicate putative CpG islands, defined as the colocalization of sites for (a) K, M, N; (b) K, M, P, 10 S, Sf; (c) K, M, P, Sf; (d) K, N, S; (e) K, N, S; (f) B, N, S; (g) B, K, N, Sf.

The pleomorphic adenoma gene region is indicated by a gray 15 shaded box.

Figure 2.

Physical contig and STS marker map in distal human 8q12. The upper line indicates the chromosome, with the 20 telomere to the left and the centromere to the right. DNA markers and genes are ordered across the top of the figure. Genes are underlined and previously known markers are indicated in bold. YAC clones and cosmid CEM3 are represented as lines together with their names. YAC end clones used as STS 25 are represented by arrows.

Figure 10.

STSs used to generate the 300 kb cosmid contig mapping at chromosome 8q12 and encompassing PLAG1.

7. Tables

TABLE 1. Pleomorphic adenomas with chromosome 8q12 rearrangements.

Case No.	chromosome 8q12 rearrangements	Other abnormalities present
CG 580	t(8;15)(q12;q14)	No
CG 588	t(3;8)(p21;q12)	No
CG 644	t(3;8)(p21;q12)	No
CG 650	t(5;8)(p13;q12)	Yes
CG 666	der(8;11)t(8;11)(p23;q13) inv(8)(p22q12)	Yes
CG 682	der(3)del(3)(p23-24)del(3)(p21.3p14.1), der(8)dir ins(8;3)(q12;p21.3p14.1)	No
CG 714	ins(8;7)(q11-12;p21p14)*	Yes
CG 787	der(8)del(8)(p11)t(8;13)(q12;q13) del(13)(q14), der(13)t(8;13)(q12;q13)	Yes

* the interpretation of the karyotype based on FISH is as follows:
 der(7)ins(7;8)(p22;q11.1q11.2) (wcp 7+, wcp 8-, Y943G4+),
 der(8)ins(8;7)(q11.2;p21p14) (wcp7+, wcp8+, Y943G4+)

TABLE 2: Analysis of YAC clones in the centromeric contig

CEPH code	Size (kb)	Landm. left	Acc. No.	Landm. right	Acc. No.	Chimeric
518G7		CH131				Y (left)
518G12		CH132				ND
770B2	1410	CH281		CH286		ND
878F2	800	CH282		CH287		ND
255H10	390	CH273		CH272		ND
531D4	430	CH274		CH277		ND
15H11	430					ND
291D12	410	CH29		CH34		Y (left)
47E9	370					ND
26F6	460					ND
916F12	1230	CH122		CH128		ND
388D6	430	CH69		CH100		ND
297E11	350	CH280		CH285		ND
253H7	160	CH279		CH284		ND
143D5	260	CH278		CH283		ND
391H2	620					Y
392A6	600					Y
84E12	500	CH289		CH290		ND
166F4	700	CH67		CH73		ND
164H5	375	END2		END6		ND
206F11		END3		END7		Y (right)
253C7	620	END4		END8		ND
295A4		CH68		CH74		Y (left)
917E11	1380	END1		END5		ND
946B7	1370	CH123		CH129		ND
935E9	1660	CH33		CH37		ND

Note. YAC clones were isolated from CEPH YAC libraries as described under Materials and Methods. ND, not detected by methods used (FISH, YAC end rescue, restriction enzyme mapping). GenBank accession numbers are given.

TABLE 3: 8q12 STS primer sequences, annealing temperatures and expected PCR product sizesAmplimers on published sequences centromeric contig

STS name	Nucleotide sequence 5' - 3'	Product size (bp)	T _a (°C)
LYN	GGAAGGAAAGGAAAGGAGA GGTTGGGTGTTGGTGTG	194	60
MOS	GAGCTAACGTAGCAAGGCT AACTGTCCTCCAGTGCAG	203	60
PENK	TAATAAAGGAGGCCAGCTATG ACATCTGATGTAAATGCAAGT	75-83	58
D8S96	TCTCTACCTCGACATACTCCTGC GTCAGAAGTGCCAATCAGACTG	113	60
D8S108	CAAACCTTGAATTACAAACAG TGTTAATATTAGACCACCTTC	116	58
D8S125	TTCTGTTCTGGTCTCAGTAGC CACATGCATATGTGTATGTGTGTG	144	62
D8S165	ACAAGAGCACATTAGTCAG AGCTTCATTTCCTCTAG	138-152	58
D8S166	GATTGTGTCATTGCACTCCA ACAAGGAAGTTCTTTGG	116	58
D8S285	GCATCACACAGAACATTG ATGGGTTATGGCCTTAC	108-124	60
D8S1069	AGCACAGTGGATATTTAGGC GGGGCTCACACAGAAGTTAA	221	60

D8S1516	GTCCCCCATCAACATGCTG CTCATCTTGTTCATAGTGTCC	174	60
D8S1661	TCTCATGCATGTTCCCTGTTG GTTGGGGTCATTAAACACTAGTCA	126	60
D8S1816	TGCACCCCTAAAAAGCATCG ACTTGCACACATGGGATCAC	143-147	60
D8S1828	AGTGCTGTTTACTTCTGTACG GCAAGACTCTGTCTCAGGA	198-238	60
AFMB055WG9	CTCCCAACCCACCCGAC TGAAAACCATAATCTCTGATGTTGC	218	60

Amplimers on newly isolated STSs centromeric contig

STS name	Nucleotide sequence 5' - 3'	Product size (bp)	T _a (°C)
CH33	CCTTGGCTGGGTTATA GGCCTATGAAGCAAGAGAG	168	60
CH34	GTACCCAGAAGGCAAGTAA GTGAAAAGGCAGAAATTAG	145	60
CH37	TTGCATGAGAATGGAAATG GGCGTTACTTCCTTTGT	176	58
CH69	AGTGCTTACAATAGGGTGAG CCATCCAGAAAGACCATAAT	336	60
CH122	TTTGTCTTGATTTTATGG TGACCAACATACTGCCTAGT	250	57
CH129	CTGAATCCCAGAACATATA AGGGTAAGTATGTCCTTTAA	110	60

CH273	ATAATGTTGAGACTTGAGA AAATGTTATCCTAATTGTA	157	58
CH274	CAGGTGAGTGGATGGTGTAA CAAGGGAGACCAAATCATC	241	60
CH277	AATGGCTATGAGGTTGTTT CACATCCTTCATTTAGCA	122	58
CH280	GGGCTGATGTTCCATTAACT GCTTCAACACCAAAAAATGCT	163	58
EM76	CTGGGAAGAGATCAAAATTC TAAAGAGACAGCACCACAAA	220	60
EM156	AGTAGCAGCAGCAACAGTCA TGCCTATTCAAGAGAAGATG	160	60
EM216	CAGTCAGTCCAGAGGTCAATT TAGGGAGGGCTTAATAGTGT	255	55?
END1	GCTCACTTCACTCCTACCC CAACCAACCACTAAAAACG	161	60
END2	GTGATTTACAGCCATT TGTAATTTCAACCAGAAG	91	50
TC65.2	TACAAACCGGGAGAAAACAG TTACAGCATTCCGATTTG	232	58

Amplimers on published sequences centromeric contig

STS name	Nucleotide sequence 5' - 3'	Product size (bp)	T _a (°C)
CYP7	TTGACTTTAAATATGATAGGTAT ACTTTTATTCTGAAAGATGAATCA	1600	55
D8S260	AGGCTTGCCAGATAAGGTTG GCTGAAGGCTGTTCTATGGA	187-213	60
D8S507	TTCCTCAGAGCAGTTCAAAG TAATCTTGCCCCAGTGAGAG	155-169	60
D8S1113	ATGCAAAGATGAACCAGGAA CCCTGGACTCATGGTACTTG	216	62
D8S1505	GGATTAAAGTTCTACAAAGGGA ACAATTCCATGAAGTGTTCACC	328	58
D8S1723	AGCTCAATGGCACGTCCTT ACTGCTGACTCAGAGCCTGG	235-243	60

Amplimers on newly isolated STSs telomeric contig

STS name	Nucleotide sequence 5' - 3'	Product size (bp)	T _a (°C)
CH31	TCACAGAATAATACAGGAT GATCACTGATGATACTAGG	220	58
CH32	ATTTGCCTCAGTGTGCAG ATTTTCAGGAGGTCAGGGA	245	62
CH35	CAAAATGACTTATGCTGAA TCTATACAGGGCATTGTGA	165	60

EM73	AAAGCAAGACCCTGTAAAGC CTTGGGCTCTATTTGTGAA	351	60
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EXAMPLE 2**5 1. Introduction**

In this example the identification of a member of the PLAG gene family that appears to be of pathogenetical relevance is described. In previous experiments, it was found that the segment between MOS and PENK on human chromosome 10 8q12 harbors the recurrent chromosome t(3;8) (p21;q12) breakpoint frequently found in pleomorphic adenomas of the salivary glands. Using a positional cloning approach, the PLAG1 gene was identified and its genomic organization characterized.

15 The largest cytogenetic subgroup of pleomorphic adenoma of the salivary glands carries chromosome 8q12 aberrations with 3p21 as preferential translocation partner. Here we demonstrate that the t(3;8) (p21;q12) results in promoter swapping between PLAG1, a novel, developmentally regulated 20 zinc finger gene on 8q12, and the constitutively expressed gene for β -catenin (CTNNB1), a protein interface functioning in adherens junctions and the WG/WNT signalling pathway. Fusions occur in the 5'-non-coding parts of both genes, exchanging regulatory control elements while preserving the 25 coding regions. Due to the t(3;8) (p21;q12), PLAG1 is activated and expression of CTNNB1 down-regulated. Activation of PLAG1 was also observed in an adenoma with a variant translocation t(8;15). The results indicate that PLAG1 activation due to promoter swapping is a crucial event in salivary 30 gland tumorigenesis.

Recently, molecular insight was obtained into the genetic basis of benign tumors by the discovery of a common genetic denominator in tumors of different types but with a particular cytogenetic profile (Schoenmakers et al. (1995); 35 Ashar et al. (1995)). Molecular analysis of recurrent chromosome 12q13-15 aberrations in pleomorphic adenoma of the salivary glands, lipoma, uterine leiomyoma, hamartoma of lung and breast, fibroadenoma of the breast, angiomyxoma,

and endometrial polyps revealed the consistent involvement of the high mobility group protein gene HMGIC; the first "benign oncogene".

Evidence is mounting that the same is likely to hold true for the closely related family member HMGIV, which is located on the short arm of chromosome 6. Both genes belong to the high mobility group (HMG) protein gene family (Bustin et al. (1990)). Their corresponding proteins possess three amino-terminal AT hooks, through which the proteins are assumed to bind to A/T-rich DNA sequences, and an acidic tail in the carboxy-terminal region. Functionally, they act as architectural factors in the nuclear scaffold, critical for the correct assembly of stereospecific transcriptional complexes (Wolffe (1994)).

The genetic aberrations frequently result in the separation of the three AT hooks from the acidic tail, although all the pivotal changes in the high mobility group protein genes that can lead to aberrant growth control are not yet known. It should be noted that a variety of chromosomal segments can act as translocation partner of 12q13-15 but each tumor type seems to have a preferential translocation partner. At present, only the preferential t(3;12) (q27-28;q15) translocation of ordinary lipoma has been characterized (Petit et al. (1996)). The gene affected on chromosome 3 is the LPP gene, which encodes a protein containing three LIM domains. LIM domains have been found in a growing number of proteins in mammals, amphibians, flies, worms, and plants and act as modular protein-binding interfaces (Schmeichel & Beckerle (1994)). LIM proteins mainly have a function in cell signalling and developmental regulation and include, for instance, transcription regulators, proto-oncogene products, and adhesion plaque constituents. The preferential t(3;12) in lipoma results in the formation of an HMGIC/LPP fusion transcript encoding a hybrid protein consisting of the three DNA binding domains of HMGI-C and the LIM domains of the protein encoded by LPP.

In an approach to systematically unravel the molecular pathogenesis of benign tumors, the inventors have focused on

other cytogenetic subgroups of these, since their mere existence indicate the presence of additional critical genes. It is of interest to establish whether such genes are functionally similar or different as compared to the high 5 mobility group protein genes HMGIC and HMGIV. In this context, the inventors have now molecularly characterized the largest cytogenetic subgroup of pleomorphic adenomas. This type of neoplasm is an epithelial tumor occurring primarily in the major and minor salivary glands. It is by far the 10 most common type of salivary gland tumor, accounting for almost 50 % of all tumors in these organs (Seifert et al. (1990)). Pleomorphic adenomas are almost exclusively benign tumors, which only rarely undergo malignant transformation. They show a marked histological diversity with epithelial 15 and myoepithelial cells arranged in a variety of patterns in a matrix of mucoid, myxoid, chondroid and, on rare occasions, even osteoid tissues. Cytogenetically, pleomorphic adenomas are characterized by recurrent rearrangements, in particular reciprocal translocations with 20 consistent breakpoints at 3p21, 8q12 and 12q13-15 (Mitelman (1994)). In addition to the cases with abnormal karyotypes, there is also a subgroup of tumors with apparently normal karyotypes (30 to 50% of the cases). Abnormalities of 8q12 are most common and are found in about 60% of the cases with 25 abnormal stemlines, whereas rearrangements of 3p21 and 12q13-15 are found in about 30% and 20% of the cases, respectively. The most frequent and characteristic abnormality so far observed in pleomorphic adenomas is a reciprocal t(3;8) (p21;q12) translocation (Mark et al. 30 (1980)). While chromosome 3p is the preferential translocation partner, most human chromosomes have been found as translocation partner of 8q12. Rearrangements of 8q12 are often found as the sole anomalies, indicating that they represent primary cytogenetic events of possible 35 pathogenetic importance.

Here, the inventors describe the positional cloning of the 8q12 translocation breakpoint as well as the identification and characterization of the genes on chromosome 8q12

and 3p21 disrupted by the t(3;8) translocation. The gene on chromosome 8q12 is a novel zinc finger gene, which we have designated PLAG1, whereas the gene on 3p21 is CTNNB1 which is the translocation partner, the gene for β -catenin, a 5 protein with an established role in cell adhesion and signal transduction (Peifer (1993)). By Southern blot and RACE analysis, we pinpoint the breakpoints within the introns of the 5'-noncoding regions of both genes and show that well-defined fusion transcripts are expressed in the tumor cells. 10 We also demonstrate that the t(3;8) translocation results in specific disruption of the transcriptional control of the two genes, leading to activation of PLAG1 and down-regulation of CTNNB1. A pleomorphic adenoma with 8q12 but not 3p21 involvement has also been studied to evaluate the 15 phenomenon of disruption of transcriptional control in a broader perspective.

2. Materials and methods

2.1 Tumor material and chromosome analysis

20 Primary pleomorphic adenomas of the salivary glands were obtained from patients at the time of surgery. Primary cultures and chromosome preparations were made and analyzed as described by Röijer et al. (1996). Primary pleomorphic adenomas, including CG368, CG580, CG644, CG752, CG753 and 25 T9587, which all carry the recurrent t(3;8)(p21;q12) as the sole anomaly, were selected for molecular analysis, as well as CG682, showing an ins(8;3)(q12;p21.3p14.1), and CG588 which carries a t(8;15)(q12;q14). CG tumors are obtained from Department of Pathology, Göteborg University, 30 Sahlgrenska University Hospital, S-41345 Göteborg, Sweden. The tumor identified as T9587 is obtained from University Hospital Ghent, Belgium. FISH analysis was performed as previously described (Röijer et al. (1996)). Slides were examined in a Zeiss Axiophot epifluorescence microscope 35 using the appropriate filter combinations. Fluorescence signals were digitalized, enhanced and analyzed using the ProbeMaster FISH image analysis system (Perceptive Scientific Instruments, Houston, Texas). Color prints were

produced using a Kodak XL 7700 monochrome continuous print r.

2.2 Preparation and analysis of DNA and RNA

5 Extraction of genomic DNA and Southern blot analysis were performed as described previously (Schoenmakers et al. (1994)). Total RNA was extracted from biopsy samples using the TRIZOL (Gibco/BRL) method and Northern analysis was performed according to standard procedures (Sambrook et al. 10 (1989)). DNA from YACs, cosmids, PCR products, and oligonucleotides was labelled using a variety of techniques. For FISH, cosmid clones or inter-Alu PCR products of YACs were biotinylated with biotin-11-dUTP (Boehringer) by nick translation. For filter hybridizations, probes were radio- 15 labelled with α -³²P-dCTP using random hexamers (Feinberg & Vogelstein (1984)). In case of PCR-products of T-genes of the invention smaller than 200 bp in size, a similar protocol was applied, but T-gene specific oligonucleotides were used to prime labelling reactions. Oligonucleotides 20 were labelled using γ -³²P-ATP.

2.3 YAC, cosmid, phage, and cDNA libraries

YAC clones in this paper were isolated from the CEPH mark 1 YAC library (Albertsen et al. (1990)), using a 25 combination of PCR-based screening and colony hybridization analysis (Green & Olson (1990)). YAC DNA was isolated and characterized as described before (Schoenmakers et al. (1995); Schoenmakers et al. (1994)). Cosmid clones were isolated from an arrayed human chromosome 8-specific cosmid 30 library (Wood et al. (1992)) obtained from Los Alamos National Laboratory (LANL). LANL-derived cosmid clones are indicated by their unique microtiter plate addresses. Phage clones were derived from a genomic library constructed with Li-14/SV40 DNA (Schoenmakers et al. (1994)) in λ FIXII 35 according to standard procedures. Cosmid and phage DNA was extracted using standard techniques involving purification over Qiagen tips (Qiagen). Positive cDNA clones were identified in a mixed poly-dT/random-primed library in λ gt11

constructed from human fetal kidney (Clontech). Library screening was performed by plaque hybridization using various DNA probes, derived from subsequently isolated cDNA clones, according to the manufacturer's instructions.

5

2.4 DNA sequencing and computer analysis

Nucleotide sequences were determined according to the dideoxy chain termination method using the T7 polymerase sequencing kit of Pharmacia/LKB or the dsDNA Cycle Sequencing System (GIBCO/BRL). Sequencing results were analyzed using an A.L.F. DNA sequencer™ (Pharmacia Biotech) on standard 30 cm, 6% Hydrolink®, Long Range™ gels (AT Biochem). Sequence analysis utilized Lasergene (DNASTAR) and BLAST and BEAUTY searches (NCBI).

15

2.5 PCR amplification of genomic DNA

PCR amplifications were carried out essentially as described before (Schoenmakers (1994)). The following amplimers were used to generate a PLAG1 exon 1 probe (5'-CAA 20 TGG CTG CTG GAA AGA GG-3' and 5'-CCC GTC CGC CGC CTC TAC ACC-3'), a PLAG1 ORF probe (5'-CGT AAG CGT GGT GAA ACC AAA C-3' and AGG GTC GTG TGT ATG GAG GTG A-3'), a PLAG1 3'-UTR 25 probe (5'-ACA TGG CAT TTC GTG TCA CT-3' and 5'-CCA CAA TGG CTC TAG AT-3') and a CTNNB1 exon 1 probe (5'-TGT GGC AGC AGC GTT GGC CCG GC-3' and 5'-CTC AGG GGA ACA GGC TCC TC-3').

2.6 Rapid amplification of cDNA ends (RACE)

Rapid amplification of 3' cDNA-ends (3'-RACE) was performed using a slight modification of part of the 30 GIBCO/BRL 3'-ET protocol. For first strand cDNA synthesis, adapter primer (AP2) AAG GAT CCG TCG ACA TC(T)17 was used. For both initial and secondary rounds of PCR, the universal amplification primer (UAP2) CUA CUA CUA CUA AAG GAT CCG TCG ACA TC was used as "reversed primer". In the first PCR round 35 the following specific "forward primers" were used: i) 5'-CAA TGG CTG CTG GAA AGA GG-3' (exon 1) or ii) 5'-AGA ATT TGG GCC TCA GAC AAG ATA-3' (3'-UTR, exon 5). In the second PCR round the following specific forward primers (nested primers

as compared to those used in the first round) were used: i) 5'-CAU CAU CAU CAU GGC CGG AGG GAG GAT GTT AA-3' (exon 1) or ii) 5'-CAU CAU CAU CAU ATT GTC CTG GGT TGA TTA TGC AT-3' (3'-UTR, exon 5). CUA/CAU-tailing of the nested, specific 5 primers allowed the use of the directional CloneAmp cloning system (GIBCO/BRL).

For 5'-RACE experiments, the Marathon cDNA Amplification kit (Clontech) was used according the manufacturer's instructions with minor modifications. The 5'-untranslated 10 end of the normal PLAG1 transcript as well as the chimeric transcripts were isolated by 5'-RACE. First strand placenta or adenoma cDNA respectively, was synthesized from 5 µg total RNA using the MV2 primer (5'-CTG CAC TTG ACC CAC CCC TTG GAT-3') located in exon 5. The ds cDNA was ligated to 15 the adaptor and amplified using the anchor primer AP1 and the MV5 primer (5'-CAG GAG AAT GAG TAG CCA TGT GC-3') also located in exon 5. A second round of PCR was performed using the anchor primer and the MV6 primer (5'-TGC ACT TGT AGG GCC TCT CTC CTG-3') located in exon 4. The final PCR products 20 were purified out of agarose gel and cloned into the pCRII vector (Invitrogen).

2.7 RT-PCR

Total RNA (5 µg) was reverse-transcribed using Super- 25 script II reverse transcriptase (GIBCO BRL) and oligo d(T) primers according to the recommended conditions. 0.25 µg of the resulting cDNA was subject to amplification using a variety of primer sets. The amplification conditions for the CTNNB1/PLAG1 fusion transcripts were 30 cycles at 94 °C for 30 10 sec and 68 °C for 1 min in a final volume of 50 µl using the Expand long template PCR system (Boehringer Mannheim). The first round PCR was carried out with the CTNNB1 primer 5'-TGT GGC AGC AGC GTT GGC CCG-3' (CAT-UP) and the PLAG1 primer 5'-CAG GAG AAT GAG TAG CCA TGT GC-3' (MV5). The 35 second round was performed on a 20 fold diluted sample with the CTNNB1 primer 5'-ACG GAG GAA GGT CTG AGG AGC AG-3' (NECAT-UP) and the PLAG1 primer 5'-TGC ACT TGT AGG GCC TCT CTC CTG-3' (MV6). To amplify the reciprocal PLAG1/CTNNB1

fusion transcript two rounds of PCR amplification were performed with 30 cycles at 94 °C for 30 sec, 63 °C for 30 sec and 72 °C for 1 min in a final volume of 50 µl. The first round was carried out with the PLAG1 primer 5'-CAA TGG 5 CTG CTG GAA AGA GG-3' (START-UP) and the CTNNB1 primer 5'-AAG GAG CTG TGG TAG TGG CAC-3' (CAT3). The second round was performed on a 20 fold diluted sample with the PLAG1 primer 5'-GGC CGG AGG GAG GAT GTT AA-3' (START-RACE) and the CTNNB1 primer 5'-GCC GCT TTT CTG TCT GGT TCC A-3' (CAT3NEST).

10

3. Results

3.1 The chromosome 8q12 breakpoint cluster region in pleomorphic adenoma

As part of a positional cloning effort to isolate the gene(s) affected by the t(3;8)(p21;q12) in pleomorphic adenomas, we constructed a yeast artificial chromosome (YAC) contig consisting of 34 overlapping YACs, spanning about 2 Mb within band 8q12 (to be published elsewhere). A long range STS and rare cutter physical map of this region was also developed. In previous experiments the t(3;8) breakpoint was mapped to a 1 Mb region flanked by MOS as proximal and by D8S166 as distal marker. One YAC within this region was shown to span the t(3;8) breakpoint in two tumors (CG588 and CG644). To further narrow down the breakpoint region we isolated cosmids corresponding to landmarks within this and other YACs in our contig (Fig. 3A). By FISH, we could demonstrate that the breakpoints in four out of four adenomas tested (CG580, CG588, CG644 and CG682) clustered within a 300 kb subregion located between MOS and STS CH129 (Fig. 3A). Two cosmids from this region, CEM23 and CEM48, were shown to span the breakpoints in adenomas CG644 (Fig. 3B) and CG682 (data not shown), respectively. To refine the distribution of breakpoints within this 300 kb subregion, we developed a contig consisting of 29 phage and cosmid clones (Fig. 3A).

3.2 Identification and characterisation of the PLAG1 gene in the 8q12 breakpoint region

While initiating experiments to identify candidate genes mapping within the 300 kb genomic segment harbouring the adenoma breakpoints, BLAST searches (Altschul et al. (1990)) of newly obtained STSs mapping within the 300 kb 5 contig revealed that the right end (CH283) of YAC clone 143D5 displayed sequence identity with a publicly available EST (expressed sequence tag) (GenBank Accession number D59273). Its position in the contig raised the possibility that this EST belonged to the candidate pleomorphic adenoma 10 gene (PLAG1), we were searching for.

In initial Northern blot experiments using a PCR probe corresponding to this particular YAC end, a 7.5 kb transcript was detected. A combination of sequential screenings of a human fetal kidney cDNA library as well as 5'- 15 and 3'-RACE experiments led to the isolation of a composite cDNA of 7313 nucleotides (GenBank accession number U65002). This cDNA contains an open reading frame (ORF) of 1500 bp starting with the ATG at position 481-483 (Fig. 4A). An in frame stop codon (TAG) is present 9 nucleotides upstream of 20 this ATG. The deduced amino acid sequence reveals seven canonical C2H2 zinc finger domains (Fig. 4B) and a non-finger region of 259 amino acid residues representing the carboxy-terminus of the deduced protein. The zinc finger 25 motifs including the linker sequences are between 28 and 35 amino acids long. The cysteine (C) and histidine (H) residues are present in their characteristic positions in each finger. The typical phenylalanine (F) and leucine (L) residues in finger 4 and 6 are lacking. Strictly spoken, the deduced protein is not a Kruppel zinc finger protein, since 30 it does not contain the characteristic H/C linker (consensus sequence TGEKPYK) in between the zinc fingers (Bellefroid et al. (1989)). Only the seven amino acids between finger 1 and 2 resemble the H/C linker (TGERPYK). The amino-terminal 35 region contains two nuclear localization signals (KRKR and KPRK). The carboxy-terminus is serine-rich (45 amino acid residues out of 259, i.e 17%), raising the possibility of a regulatory role that may be controlled by serine/threonine kinases.

Outside the ORF, some interesting features in the cDNA can be observed. In the 3' untranslated region (UTR), which is 5333 bp long, a polyadenylation signal is present starting at position 7297, and there is also a TG-repeat, which 5 might be of regulatory relevance. In addition, there is an ATTTA sequence motif, which has previously been shown to mediate mRNA destabilization in certain lymphokine and immediate early genes (Sachs (1993)). The ATTTA motif is repeated twelve times in the 3'-UTR.

10 No sequence similarity was found to any of the known zinc finger proteins but preliminary studies have indicated that the human genome contains at least one additional gene that is closely related to our candidate pleomorphic adenoma gene. Nine anonymous ESTs (expressed sequence tags) with 15 sequence similarity to sequences of our new gene were found in public databases and these ESTs could be aligned resulting in a contiguous sequence of about 1 kb. An open reading frame appeared to be present with coding potential for protein sequences with zinc-finger-like features. Sequence 20 identity between the aligned ESTs and our new gene appeared to be about 75% at the amino acid sequence level; i.e. in the region of our gene encoding zinc fingers 4 to 7. Of interest to note furthermore is that these anonymous ESTs sequences have been mapped to chromosome 6q24, which is 25 implicated in tumors of the salivary glands.

3.3 Genomic organization of the PLAG1 gene

Comparison of transcribed and genomic DNA sequences of the PLAG1 gene revealed that it contains 5 exons (Fig. 3A). 30 The transcriptional orientation of the gene is directed towards the centromere. The first three exons are noncoding, the fourth exon contains the translation start site (ATG), and the amino-terminus of the protein including one complete zinc finger domain. The second finger is split by intron 4 35 and continues into exon 5, which contains the remaining part of the ORF and the long 3'-UTR (5533 bp). Based on our YAC and cosmid maps, the PLAG1 locus spans about 35 kb, with a large intron (approximately 25 kb) between exon 1 and exon

2. 3'-and 5'-RACE analysis revealed the existence of two alternatively spliced mRNAs which differ from each other by the presence or absence of the noncoding exon 2. The difference in electrophoretic mobility of the two mRNA isoforms 5 is too small to distinguish them by Northern blot analysis. The functional relevance of this alternative splicing remains to be elucidated.

3.4 Rearrangements of PLAG1 in primary pleomorphic adenoma 10 In an attempt to establish whether the 8q12 aberrations in primary pleomorphic adenomas with t(3;8)(p21;q12) led to rearrangements in PLAG1, Southern blot experiments were performed using various PLAG1-derived probes. With a probe within the ORF (STS probe EM265) or the 3'-UTR (KK64) of 15 PLAG1, no rearrangements were detected. However, using probes corresponding to the 5' noncoding region of PLAG1, i.e. intron 1 (STS probes EM416 or EM317) or exon 2 (STS probe EM440), rearrangements were detected in each of the t(3;8) tumors tested (data not shown). Similar Southern blot 20 analysis of primary pleomorphic adenoma CG580, which carries a t(8;15)(q12;q14), revealed also a rearrangement in the 5' noncoding region of PLAG1 (Fig. 5). These results seem to directly implicate PLAG1 as a critical gene in pleomorphic adenoma tumorigenesis. Furthermore, the mechanism might 25 involve transcriptional deregulation of PLAG1, since the rearrangements in PLAG1 were invariably found in the 5' noncoding region of PLAG1 leaving its coding region intact.

3.5 t(3;8) results in promoter swapping between PLAG1 and 30 the gene for β -catenin, CTNNB1

The observation that the 8q12 translocation breakpoints in various primary tumors were mapped to intron 1 or intron 2 of the 5'-noncoding region of PLAG1 raised the possibility that the t(3;8) results in the production of a chimeric 35 transcript consisting of PLAG1 sequences fused to those of a gene located on chromosome 3p21. To test this possibility, 5'-RACE experiments were performed using total RNA of the primary tumors CG644 and CG682, which both carry a 3;8-

r arrangement. We used a prim r specific for exon 5 (MV2) in the cDNA synthesis. Following ligation of an adaptor to the cDNA, PCR amplification was performed using, in the first round, an adaptor-specific primer and a PLAG1-specific 5 primer corresponding to sequences of exon 5 (MV5) and, in a second round, nested adaptor-specific primer and PLAG1-specific primer corresponding to sequences of exon 4 (MV6). Nucleotide sequence analysis of the PCR product revealed that the ectopic sequences were fused to the acceptor splice 10 site of exon 3 of PLAG1. BLAST analysis revealed that they were identical to exon 1 sequences of CTNNB1 (Nollet et al. (1996)), the gene for β -catenin, which has previously been assigned to chromosome 3p21 (Kraus et al. (1994)).

Independent evidence for the involvement of CTNNB1 in 15 the t(3;8)-rearrangements in pleomorphic adenomas was obtained by FISH using two YACs (756G5 and 750D3), which contain the complete gene for β -catenin. According to published maps YAC 756G5 is contained within the proximal part of mega-YAC 750D3 (Gemmell et al. (1995)). As expected, both YACs 20 spanned the 3p21 breakpoint in adenoma CG682, with hybridization signals on the normal 3, the der(3) and the der(8) (data not shown). Detailed analysis of a few prometaphase cells enabled us to sublocalize the β -catenin locus to band 3p21.3, which is in accordance with previous 25 mapping data.

To extend our observation to a larger number of tumors, we applied a reverse transcription-polymerase chain reaction (RT-PCR) approach. RT-PCR amplifications using primers specific for CTNNB1 and PLAG1 were carried out with RNA from 30 tumors CG368, CG588, CG644, CG752, CG753 and T9587, which all carry the recurrent t(3;8), and from tumor CG682, which carries an ins(8;3)(q12;p21.3p14.1). RNA from tumor CG580, which carries a t(8;15), was included as a negative control. PCR experiments resulted in the generation of PCR products 35 corresponding to hybrid transcripts consisting of PLAG1 and CTNNB1 sequences, in seven out of seven t(3;8) tumors analyzed (Fig. 6). In tumors CG368, CG588, CG682, CG752, and T9587, PCR products of 509 bp (Fig. 6A, PCR product A) and

614 bp (Fig. 6A, PCR product B) were generated, whereas in tumors CG644 and CG753, only the PCR product of 509 bp was found. The PCR product of 509 bp (from NECAT-UP up to MV6) is corresponds to a hybrid transcript containing exon 1 of 5 CTNNB1 and exons 3 to 5 of PLAG1. The PCR product of 605 bp contains an extra 105 bp, which corresponds to the alternatively spliced exon 2 of PLAG1. It points towards the presence of a related isoform consisting of exon 1 of CTNNB1 and exons 2 to 5 of PLAG1. This was also confirmed by nucleotide sequence analysis of the PCR products. We were also able to demonstrate that the corresponding reciprocal fusion transcripts are expressed. In all tumors except CG682, a PCR product of 130 bp was generated corresponding to a fusion transcript consisting of exon 1 of PLAG1 and exons 2 to 16 10 of CTNNB1. In tumor CG753, an additional PCR product was detected, corresponding to a fusion transcript consisting of exons 1 to 2 of PLAG1 and exons 2 to 16 of CTNNB1. This additional band was also observed but with weak intensity in tumor CG644. All these results indicate that in CG644 and 15 CG753, the 8q12 translocation breakpoints are located in intron 2, whereas the breakpoints of tumors CG368, CG588, CG682, CG752, and T9587 are located in intron 1. Interestingly, using 5' RACE analysis with the tumor CG580, which carries a t(8;15) translocation, we found that the break- 20 point occurs also in the same region leading to a fusion transcript with ectopic fused to exon 3 of PLAG1. 25

3.6 Activation of PLAG1 expression due to promoter swapping

Northern blot analysis was performed to evaluate the 30 effect of the t(3;8)(p21;q12) translocation on the expression levels of PLAG1 and CTNNB1 (Fig. 7). As previously mentioned, PLAG1 is expressed as a 7.5 kb transcript, which was readily detected in human fetal lung, liver, and kidney but not in fetal brain. In adult tissues, the 7.5 kb transcript 35 was not detected in human heart, brain, lung, liver, skeletal muscle, kidney, pancreas, and salivary gland. Low levels of PLAG1 expression was found in human placenta (Fig. 7A). In contrast, the CTNNB1 gene was ubiquitously expressed as a

3.8 kb RNA doublet in all tissues tested (Fig. 7B). Similar results were obtained in the Northern evaluation of fetal and adult mouse tissues (data not shown).

Northern blot analysis was performed on two pleomorphic adenomas with t(3;8) and on one tumor with a variant t(8;15) (Fig. 7C). In contrast to normal salivary gland tissue in which no PLAG1 transcripts could be detected, the three tumors expressed a 7.5 kb transcript. This transcript does not correspond to the normal PLAG1 transcript (exons 1 to 5), since an exon 1-specific probe failed to hybridize to this transcript (data not shown). Instead, a probe specific for exon 1 of CTNNB1 recognized this 7.5 kb transcript as well as the 3.8 kb doublet of CTNNB1 transcripts in the two tumors with a t(3;8). In contrast, only the 3.8 kb doublet of CTNNB1 transcripts was detected in adenoma CG580 with a variant t(8;15). The expression level of CTNNB1 in this tumor was about twofold higher as compared to tumors CG644 and CG682, which is expected since none of the CTNNB1 alleles is rearranged. Collectively, these results suggest that PLAG1 expression in these pleomorphic adenomas is driven by the 5' regulatory sequences either of the CTNNB1 gene for the cases with t(3;8) or of an as yet unknown gene on chromosome 15 for the case with t(8;15). These results indicate that the principal mechanism in pleomorphic adenomas with 8q12 rearrangements is activation of PLAG1 expression, due to promoter swapping between PLAG1 and a translocation partner gene, preferentially CTNNB1. Furthermore, expression of the translocation partner gene is likely to be down-regulated concomitantly; e.g. as demonstrated for CTNNB1.

30

4. Discussion

We have characterized a novel gene, PLAG1, which we propose is a critical locus involved in the development of pleomorphic adenoma of the salivary glands. The gene was identified on the long arm of chromosome 8, close to the MOS proto-oncogene, as a result of a positional cloning project to molecularly define the t(3;8)(p21;q12), which is the most frequent chromosome aberration in pleomorphic adenomas. We

have also identified the translocation partner gene at 3p21, i.e. the CTNNB1 gene which encodes β -catenin. In addition to 8q12 and 3p21, chromosome rearrangements involving 12q13-15 are also frequently observed in pleomorphic adenomas. The 5 high mobility group protein gene HMGIC was recently identified as the gene consistently rearranged in these cases (Schoenmakers et al. (1995)). In conclusion, we have 10 molecularly defined the three main chromosomal rearrangements implicated in the genesis of pleomorphic adenomas.

Structural rearrangement of the 8q11-13 region are consistently found also in benign lipoblastomas. These tumors are considered to be unique variants of lipoma, occurring primarily in children before 3 years of age. Among 15 the seven cases so far analyzed cytogenetically, rearrangements involving 8q11-13 were observed in six cases (Mitelman (1994)). Similar to pleomorphic adenomas, the translocation partners of 8q12 are variable. The similarities in chromosomal pattern between these two tumor types suggest 20 that PLAG1 is the target of the 8q11-13 rearrangements also in lipoblastomas.

We have also elucidated the molecular consequences of the t(3;8) translocation. The 8q12 translocation breakpoints were invariably found in the 5'-non-coding region of PLAG1 25 in all seven primary tumors tested. The breakpoints in the CTNNB1 gene were always found in the first intron. As a result of the translocation, the coding sequences of PLAG1 are brought under control of the 5' regulatory sequences of the CTNNB1 gene, and vice versa, resulting in the activation 30 of PLAG1 and down-regulation of CTNNB1. Based on their expression patterns in normal fetal and adult tissues, it is clear that the regulation of expression of the two genes is very different. The CTNNB1 gene is highly and ubiquitously expressed, whereas PLAG1 is developmentally regulated, with 35 expression restricted to fetal tissues. It should be emphasized that in adult tissues, PLAG1 is either not expressed or expressed at very low levels. In the adenomas, both the CTNNB1/PLAG1 and the reciprocal PLAG1/CTNNB1 transcripts

were detected by RT-PCR; detection of the latter transcripts sometimes required three rounds of PCR, indicating that the expression levels are very low. Our findings represent the first example of reciprocal exchange of expression control elements in solid tumors. Since the coding sequences of both genes are invariably preserved, the molecular mechanism could be classified as promoter swapping, resulting in activation of PLAG1 and down-regulation of CTNNB1 expression.

Since the t(3;8) in pleomorphic adenomas invariably leads to activation of PLAG1, it is of interest to evaluate possible physiological implications. The PLAG1 gene encodes a protein with a deduced molecular weight of 56 kDa and a deduced pI of 8.56. Analysis of the open reading frame of PLAG1 reveals seven zinc fingers in the amino-terminal region. The carboxy-terminal region is rich in serine residues. Furthermore, two potential nuclear localization signals are present (residues 22-25 and 29-32). Collectively, this suggests that the PLAG1 protein is a novel member of the large zinc finger gene family. Zinc finger motifs were originally identified as DNA binding structures in the RNA polymerase III transcription factor TFIIIA, which binds to the internal control region of the 5S RNA gene. TFIIIA-like zinc fingers are present in a variety of regulatory proteins found in higher and lower eukaryotes. This type of zinc finger motif consists of \approx 30 amino acids with two cysteine and two histidine residues (C2H2) that stabilise the domain by tetrahedrally coordinating a Zn^{2+} ion. A region of \approx 12 amino acids between the invariant cysteine-histidine pairs is characterized by scattered basic residues and several conserved hydrophobic residues. Most zinc finger genes studied so far encode polymerase II transcription factors. Apart from transcriptional modulation and control of RNA metabolism, chromatin packaging might also constitute an important activity through which zinc finger proteins exert their regulatory roles (El-Baradi & Pieler (1991)). It should also be noted that the presumed role of HMGIC is also in chromatin modelling (Schoenmakers et al. (1995); Ashar et

al. (1995); Wolffe (1994)). The mammalian genome is known to contain a large number of C2H2 zinc finger genes (Bellefroid et al. (1989)) and the number of such genes implicated in cancer is growing steadily.

5 The function of the serine rich carboxy-terminal part of PLAG1 is unknown but it may have a regulatory function that can be controlled by serine/threonine kinases. If PLAG1 encodes a DNA binding protein, as the presence of its zinc fingers suggests, the carboxy-terminal region might
10 represent the transactivation domain. At least four different primary sequence motifs that characterize the activation domains are identified thus far, i.e. acidic, glutamine-rich, proline-rich and serine/threonine-rich. They likely represent regions that functionally interact with
15 other proteins (Mitchell & Tjian (1989)).

Activation of PLAG1 due to promoter substitution may lead to deregulation of genes normally controlled by PLAG1. If overexpression of PLAG1 is indeed a major molecular consequence of the 8q12 translocations than this could
20 possibly be achieved also by other mechanisms, including for instance an increase in gene copy number. In this context it should be noted that a small subgroup of pleomorphic adenomas show trisomy 8, often as the sole anomaly (Mitelman (1994)). In addition, trisomy 8 is a common numerical
25 abnormality found in several histological subtypes of malignant salivary gland tumors as well as in different subtypes of leukemias and sarcomas (Mitelman (1994)).

The preferential fusion partner of PLAG1 is the CTNNB1 gene, which encodes β -catenin, a cytoplasmic protein of
30 about 88 kDa (Gumbiner & McCrea (1996)). Its frequent involvement in pleomorphic adenomas as found here might point towards a critical role of CTNNB1. Most likely, a role of CTNNB1 is to provide an active promoter in front of the PLAG1 gene. However, since the observed promoter swapping
35 between PLAG and CTNNB1 leads to down-regulation of CTNNB1 expression, it may also lead to other physiological consequences, especially since β -catenin is a broad-range protein interface, as that has been implicated in highly

diverse processes, such as human colon cancer, epithelial cell adhesion, embryonal axis formation in Xenopus, and pattern formation in Drosophila (Peifer (1993)). The β -catenin protein has also been found as structural component 5 of adherens junctions (AJs) (Peifer (1993); Kemler (1993); Gumbiner (1996)), which are multiprotein complexes assembled around Ca^{2+} -regulated cell adhesion molecules (cadherins). β -Catenin binds directly to cadherins and acts as a protein interface between cadherin and the cytoskeleton. The 10 cadherin- β -catenin complex mediates cell adhesion, cytoskeletal anchoring, and signalling, which are important processes for regulation of cell growth and behaviour. It has been suggested that AJ complexes may play a role in the transmission of signals for contact inhibition (Peifer 15 (1993)). Down-regulation of β -catenin, as occurs in pleomorphic adenomas, could interfere with this transmission and lead to growth deregulation. Since β -catenin levels apparently are important physiologically, it might be that down-regulation of CTNNB1 expression plays some role in 20 pleomorphic adenoma development, possibly synergistically to PLAG1. In conclusion, as activation of PLAG1 is observed in all pleomorphic adenomas with 8q12 involvement tested, this seems to constitute a major factor in salivary gland tumor formation. The critical role of down-regulated levels of β - 25 catenin remains to be established.

Previous studies of HMGIC have shown that rearrangements of this gene preferentially occur in particular cytogenetic subgroups of benign mesenchymal tumors, with the only exception so far being a subgroup of pleomorphic adenomas (Schoenmakers et al. (1995)). When considering the 30 findings in the latter type of tumors, it should be noted that, although they are epithelial in origin, they often also show a varying degree of mesenchymal differentiation. This may be explained by the fact that pleomorphic adenomas 35 are thought to originate from a pluripotent reserve cell in the terminal duct system which possesses the capacity to differentiate into both epithelial and myoepithelial cells (Evans & Cruickshank (1970)). The latter cells may function

as facultative mesenchymal cells, and are responsible for the production of extracellular material, including myxochondroid stroma (Dardick et al. (1991)). An intriguing question in this context is of course whether HMGIC is

5 preferentially affected in adenomas with a prominent stromal component while PLAG1 is preferentially affected in tumors with little or no stroma. Histological re-examination of all but one of the adenomas with PLAG1 involvement in this study, revealed that all cases contained mesenchymal

10 components, including both myxoid and chondroid tissues, suggesting that mesenchymal differentiation is not restricted to adenomas with HMGIC involvement. It should be noted that previous cytogenetic observations have already indicated that chromosome 12q13-15 and 8q12 rearrangements

15 constitute mutually exclusive pathways for the evolution of pleomorphic adenomas (Sandros et al. (1990)).

The identification and cloning of a novel "benign oncogene" activated by chromosome translocations constitute a major step towards an increased understanding of the

20 molecular pathogenesis of benign neoplastic growth. Our findings may lead to new insights into the genetic differences between benign and malignant tumors. In addition, the present findings may have diagnostic implications. The fact that PLAG1 is not normally expressed in adult tissues, but

25 activated in pleomorphic adenomas due to 8q12 rearrangements, makes it a potentially useful tumor marker in the differential diagnosis of benign and malignant salivary gland tumors. On the basis of the fusion transcripts that are formed, highly specific diagnostic assays can be developed, as will be illustrated in the following examples.

FIGURE LEGENDS

Figure 3

A: Contig of 3 overlapping YACs (bold lines), 27 cos-
35 mids and 2 phages, containing 27 landmarks and spanning a 300 kb DNA region on chromosome 8q12. Contig elements are labelled or numbered and defined in the list below. Cosmid clones isolated from the arrayed chromosome 8-specific

cosmid library constructed at Los Alamos National Laboratory (LANL) (Wood et al. (1992)) are named after their unique microtiter plate addresses. #1 and #22 are genomic phage clones; #21 is a clone isolated from the non-arrayed LANL 5 chromosome 8-specific cosmid library. The orientation of the contig on the long arm of chromosome 8 is given as well as the order of 27 landmarks. It should be noted that the contig is not scaled. Below the contig, the genomic organization and the relative location of the PLAG1 gene is 10 given schematically, with exact sizes (bp) of its exons and estimated sizes (kb) of its introns. Noncoding sequences are represented as open boxes and coding sequences as black boxes. The relative positions of the translation initiation (ATG) and stop (TAG) codons in PLAG1 are indicated. At the 15 bottom of the figure, characteristics of the deduced protein encoded by PLAG1 are given. Zinc fingers are labelled F1-F7.

1 = PEM18 ← CH33 =D8SXXX	11 = 163E1 ← CH280 =D8SXXX	21 = CEM55 ← EM195 =D8SXXX
2 = 96E9 ← PENK =D8SXXX	12 = 203B8 ← CH280 =D8SXXX	22 = PEM49 ← EM317 =D8SXXX
3 = 41E12 ← PENK =D8SXXX	13 = 50B7 ← EM187 =D8SXXX	23 = 149G12 ← D8S285
4 = 17B6 ← EM172 =D8SXXX	14 = 115H9 ← EM187 =D8SXXX	24 = 64C1 ← D8S285
5 = 93B2 ← EM172 =D8SXXX	15 = 129B10 ← CHI29 =D8SXXX	25 = 64D1 ← D8S285
6 = 47D11 ← CH280 =D8SXXX	16 = 116A2 ← CHI29 =D8SXXX	26 = 143B1 ← MOS =D8SXXX
7 = S3B10 ← CH280 =D8SXXX	17 = 7B7 ← CHI29 =D8SXXX	27 = 143H1 ← MOS =D8SXXX
8 = 66E5 ← CH280 =D8SXXX	18 = 4H4 ← EM156 =D8SXXX	28 = 148E9 ← MOS =D8SXXX
9 = 144A7 ← CH280 =D8SXXX	19 = 4H5 ← EM156 =D8SXXX	29 = 22B8 ← MOS =D8SXXX
10 = 163D11 ← CH280 =D8SXXX	20 = 4H6 ← EM156 =D8SXXX	

(clone number, cosmid or phage clone, STS, accession number)

30

B: Mapping of the 8q12 translocation breakpoint in an adenoma (CG644) with a t(3;8)(p21;q12). Cosmid CEM48 (white spots) was co-hybridized with an alpha-satellite probes one specific for chromosome 3 and the other for chromosome 8 35 (black spots). Hybridization signals were found on the normal 8, the der (3), and the der (8), indicating that CEM48 spans the t(3;8)(p21;q12) breakpoint. Chromosomes are stained in blue with DAPI.

40 Figure 4

A: cDNA and deduced amino acid sequence of PLAG1. The relative positions of exon/intron boundaries are indicated by triangles (▼). The conserved C, F, L and H residues in

the zinc finger domains are underlined. Residues 22-25 and 29-32 constitute two potential nuclear localization signals. A potential polyadenylation signal and the putative mRNA destabilizing ATTTA motifs in the 3'-noncoding region are 5 underlined. The nucleotide sequence of the complete cDNA has been deposited at GenBank under accession number XXX.

B: Alignment of the seven zinc-finger-like motifs found in the deduced amino acid sequence of PLAG1 relative to the C2H2 consensus motif. The canonical C, F, L and H residues 10 are given in bold.

Figure 5

Illustrative example of the detection of DNA rearrangement in PLAG1 of a primary pleomorphic adenoma using Southern blot analysis. DNA of pleomorphic adenoma CG368 (panel I) and control DNA isolated from normal lymphocytes (panel II) were digested with restriction endonuclease BamHI (B), EcoRI (E), HindIII (H), or PstI (P), as indicated. A probe with specificity for exon 3 of PLAG1 (EM440) was used. The 20 molecular weight markers are 23.1, 9.4, 6.5, 4.3, 2.3 and 2.0 kb, respectively.

Figure 6

A: Detection of CTNNB1/PLAG1 and PLAG1/CTNNB1 fusion 25 transcripts by RT-PCR in primary adenomas. I. CTNNB1/PLAG1 were detected using the RT-PCR protocol and primers described in detail in Methods. Primary tumors analyzed included CG368 (lane 1), CG588 (lane 2), CG644 (lane 3), CG682 (lane 4), CG752 (lane 5), CG753 (lane 6), T9587 (lane 7), and 30 CG580 (lane 8). II. PLAG1/CTNNB1 fusion transcripts were detected similarly using the same samples as under "I". Details of the primers used here are given in Methods Section. PCR products are labelled A-D.

B: Schematic representation of the nature and origin of 35 CTNNB1/PLAG1 and PLAG1/CTNNB1 fusion transcripts in primary pleomorphic adenomas with t(3;8)(p21;q12). At the top of the figure, the exon/intron distribution of the PLAG1 gene is given, at the bottom, the exon/intron distribution for the

CTNNB1 gene. Positions of chromosome breakpoints are indicated by an arrow (↖). Translation initiation sites are indicated by asterisks (*) and stop codons by triangles (▼). A-D: schematic exon compositions of hybrid transcripts, as established by 5'-RACE analysis. A: cDNA sequence junction between exon 1 of CTNNB1 and exons 3-5 of PLAG1. B: cDNA sequence junction between exon 1 of CTNNB1 and exons 2-5 of PLAG1. C: cDNA sequence junction between exon 1 of PLAG1 and exons 2-16 of CTNNB1. D: cDNA sequence junction between exons 1-2 of PLAG1 and exons 2-16 of CTNNB1.

Figure 7

A: Northern blot analysis of the expression pattern of PLAG1 in normal human fetal tissues including brain (1), lung (2), liver (3), kidney (4) as well as adult tissues including heart (5), brain (6), placenta (7), lung (8), liver (9), skeletal muscle (10), kidney (11), and pancreas (12).

B: Northern blot analysis of the expression pattern of the CTNNB1 gene in normal human fetal and adult tissues as described under "A".

C: Detection of CTNNB1/PLAG1 transcripts in pleomorphic adenomas by Northern blot analysis using exon 1 of CTNNB1 as a molecular probe. Lane 1, RNA of CG644 (t(3;8)), lane 2, RNA of CG580 (t(8;15)), and lane 3, RNA of CG682 (ins 3p21 (8q12)). The CTNNB1/PLAG1 fusion transcript is indicated.

D: Using the same blot as under "C", detection of CTNNB1/PLAG1 transcripts using a probe with specificity for the 3' UTR of PLAG1 (probe KK64). The CTNNB1/PLAG1 transcript is indicated.

EXAMPLE 3

The discovery of PLAG1 made it possible to identify PLAG1-related genes. The possibility that the human genome contains such genes was raised by Southern blot data showing bands (weak hybridization signals). In this example, the identification of another member of the PLAG gene family

that might be of pathogenetical relevance is described, i.e. the PLAG2 gene.

To isolate cDNA clones corresponding to other members of the PLAG1 gene family, a cDNA library in λ ZAP was used 5 (Stratagene). As molecular probe, the complete human PLAG1 cDNA was used. Screening of the cDNA library was performed according to routine procedures using low stringency hybridization conditions (Schoenmakers et al. (1994)). This resulted in the isolation of a number of overlapping cDNA 10 clones presumably corresponding to another member of the PLAG family. From the inserts of these, a 2.7 kbp composite cDNA could be constructed that contained an open reading frame (1233 nucleotides) for a protein (411 amino acids) structurally similar to PLAG1. This protein was designated 15 PLAG2. The 2.7 kbp human PLAG2 cDNA fragment started 176 nucleotides upstream of the ATG start codon.

The nucleotide sequence of the composite PLAG2 cDNA and the deduced amino acid sequence of the corresponding PLAG2 protein are shown in Figure 8. Nucleotide sequences were 20 determined according to the dideoxy chain termination method using the T7 polymerase sequencing kit of Pharmacia/LKB. DNA fragments, subcloned in the pBLUESCRIPT or pGEM-3Zf(+) vector, were sequenced using standard primers and primers synthesized based upon newly obtained sequences. Nucleotide 25 sequence data were obtained from both strands and analyzed using the sequence analysis computer programs Genepro (Riverside Scientific), PC/Gene and Intelligenetics (IntelliGenetics, Inc.).

To establish that the isolated PLAG2 sequences corresponded to expressed sequences, Northern blot analysis was 30 performed according to routine procedures. Total RNA was extracted from various human tissues and cell lines using TrizolTM (GIBCO/BRL). After precipitation, concentrations of RNA in the samples were estimated via spectroscopic determination. 35 40 μ g of total RNA from each sample was run on 1% agarose gels containing 1 x MOPS (0.02 M MOPS (Sigma) pH 7.0, 50 mM Na-acetate, 10 mM EDTA pH 8.0), and 0.66 M formaldehyde (Sigma) in a 1 x MOPS buffer for 16 h at 4 V/cm.

After gel electrophoresis, RNA was transferred to Hybond N⁺ filters (Amersham) using 10 x SSPE (20 x SSPE: 3.0 M NaCl, 0.16 M NaH₂PO₄ pH 7.4, 0.02 M EDTA) for standard capillary blotting. After completion of the transfers, nylon filters 5 were irradiated with ultraviolet light using a Stratalinker (Stratagene). Prehybridizations of the blots were carried out for 3 h at 42 °C in hybridization buffer consisting of 5 x SSPE, 10 x Denhardt's, 2% SDS, 50% formamide and 100 µg/ml herring sperm DNA. For hybridizations, blots were incubated 10 overnight in the same buffer as described for the prehybridizations. Subsequently, blots were washed for 40 min at room temperature in 2 x SSC containing 0.05% SDS and for 40 min in 0.1 x SSC containing 0.1% SDS at 50 °C. Autoradiographs 15 were obtained by exposing Kodak X-AR film at -80 °C with intensifying screen.

In Northern blot analysis using the complete 2.7 kb PLAG2 cDNA as molecular probe and rather stringent hybridization conditions, four distinct mRNAs were detected. These varied in size and included mRNAs of about 4.4 kb, 3.1 kb, 20 2.9 kb, and 1.9 kb. The 3.1 and 2.9 kb transcripts were preferentially seen in fetal tissue, whereas the other transcripts were seen preferentially in adult tissues. These results established the expression of the PLAG2 sequences in a variety of cell types. Furthermore, the detection of 25 various mRNAs with the PLAG2 cDNA probe suggests the existence of various isoforms and raises the possibility that various PLAG2 protein isoforms may be produced.

EXAMPLE 4

30 Detection of hybrid PLAG1 in salivary gland tumor cells.

cDNA clones of the chromosome 3-derived CTNNB1 gene were isolated and the nucleotide sequence thereof established. The nucleotide sequence data of a composite cDNA are shown in Fig. 9. The amino acid sequence of the CTNNB1-35 encoded protein was deduced.

In 5'-RACE analysis of primary pleomorphic adenomas of the salivary glands, PLAG1

containing fusion transcripts were identified. 5'-RACE experiments were performed using total RNA of the primary tumors. A primer specific for exon 5 (MV2) was used in the cDNA synthesis. Following ligation of an adaptor to the 5 cDNA, PCR amplification was performed using, in the first round, an adaptor-specific primer and a PLAG1-specific primer corresponding to sequences of exon 5 (MV5) and, in a second round, nested adaptor-specific primer and PLAG1-specific primer corresponding to sequences of exon 4 (MV6). 10 Nucleotide sequence analysis of the PCR product revealed that the ectopic sequences were fused to the acceptor splice site of exon 3 of PLAG1. BLAST analysis revealed that they were identical to exon 1 sequences of CTNNB1, the gene for β -catenin, which has previously been assigned to chromosome 15 3p21.

EXAMPLE 5**Diagnostic test for pleomorphic adenoma of the salivary glands**

20 Fine needle biopsies of patients having a pleomorphic adenoma of the salivary gland (with a chromosome 8q12 aberration others with a normal karyotype) were taken. From the material thus obtained total RNA was extracted using the standard TRIZOL™ LS protocol from GIBCO BRL as described in 25 the manual of the manufacturer. This total RNA was used to prepare the first strand of cDNA using reverse transcriptase (GIBCO/BRL) and an oligo dT(17) primer containing an attached short additional nucleotide stretch. The sequence of the primer used is as described in Example 2, under point 30 2.6 [AAG GAT CCG TCG ACA TC(T)17]. RNase H was subsequently used to remove the RNA from the synthesized DNA/RNA hybrid molecule. PCR was performed using a gene-specific primer (Example 2, point 2.6) and a primer complementary to the attached short additional nucleotide stretch. The thus 35 obtained PCR product was analysed by gel electrophoresis. Fusion constructs were detected by comparing them with the background bands of normal cells of the same individual.

In an additional experiment, a second round of hemi-nested PCR was performed using one internal primer and the primer complementary to the short nucleotide stretch [AAG GAT CCG TCG ACA TC(T)17]. The sensitivity of the test was 5 thus significantly improved.

EXAMPLE 6

Diagnostic test for uterine leiomyoma.

10 Uterine leiomyoma is the most common pelvic tumor in women, occurring with an incidence up to 77% of women of reproductive age when tumors are counted after 2 millimeter serial sectioning of consecutive hysterectomy specimens. Often multiple leiomyomas are present, with estimates as 15 high as an average of 6.5 tumors per uterus. Although most patients with these steroid-dependent tumors are asymptomatic, symptomatic leiomyomas can be associated with abnormal uterine bleeding, pelvic pain, urinary dysfunction, spontaneous abortions, premature delivery and infertility. The 20 high incidence of this benign smooth muscle tumor constitutes a major public health problem with the diagnosis of myomatous uterus leading to over 200,000 hysterectomies performed annually in the United States. Furthermore, non-random inactivation of the X chromosome has clearly demonstrated that uterine leiomyomas are monoclonal proliferations. Although leiomyomata typically are benign tumors, in a 25 very low percentage (< 0.1%) they might be able to metastasize or even progress to malignancy.

Besides a normal karyotype which is being found in 30 approximately 70% of the cases investigated, several cytogenetically abnormal subgroups, can be distinguished among leiomyomata. Leaving out of consideration the group which shows random changes, one of the largest cytogenetic subgroups (comprising approximately 25% of the cytogenetically 35 abnormal tumors) was first described in 1988 and is characterized by the involvement of 12q15 and/or 14q23-24, mainly as t(12;14)(q14-15;q23-24). Another subgroup, with a similar incidence, contains deletions involving the long arm of

chromosom 7, with region q21-22 being the most probable commonly involved region. Another subset of leiomyomas is characterized by numerical aberrations, mainly trisomy 12. This trisomy is found in approximately 10% of the cytogenetically abnormal leiomyomas. Furthermore, chromosome 6p21--pter, has been found to be recurrently involved in roughly 5% of the cases studied. Finally, a small percentage (approx. 3.5%) of leiomyomata shows t(1;2)(p36;p24). In uterine leiomyomata with chromosome 12q14-15 aberrations, the HMGIC gene is affected and in those with chromosome 6p aberrations, the HMGIV gene is affected.

The observation that 70% of the uterine leiomyomata tested show a normal karyotype prompted studies to investigate whether activation of a PLAG gene could be found in uterine tumors. Therefore, Northern blot and PCR analysis were performed.

Specimens of tumor material surgically removed from patients and classified by routine pathology as uterine leiomyoma, uterine leiomyosarcoma, fibroleiomyoma, or uterine fibroma were used. From the material thus obtained, total RNA was extracted using the standard TRIZOL™ LS protocol from GIBCO BRL as described in the manual of the manufacturer. This total RNA was used in Northern blot analysis, which revealed activation of PLAG1 in about 50% of the tumors tested, indicating that specific PLAG activation is also observed in these types of tumors. These results were confirmed by PCR analysis. These results reveal the possibilities of using PLAG1 as a diagnostic marker on routine biopsies taken from patients. Since PLAG1 is implicated in this type of tumor, PLAG1 can be used as a target for the development of novel therapeutic strategies for uterine leiomyoma.

EXAMPLE 7

35 Diagnosis of lipoblastoma.

Lipoblastomas are benign pediatric tumors that are assumed to result from proliferation of primitive adipocytes. These type of tumors often grow rapidly but there are

no reports that they metastasize. Because of the rapid growth and their histopathological features, lipoblastomas can mimic myxoid or well-differentiated liposarcoma which makes the diagnosis problematic. As a direct consequence, 5 therefore, some of these tumors have been treated in an unnecessarily aggressive manner.

A recurrent rearrangement involving the long arm of chromosome 8 has been reported for lipoblastomas. Southern blot analysis detected a rearrangement in the PLAG1 gene 10 opening the possibility of using PLAG1 as a diagnostic marker.

A biopsy of a patient having a lipoblastoma was taken. From the material thus obtained total RNA was extracted using the standard TRIZOL™ LS protocol from GIBCO BRL as 15 described in the manual of the manufacturer. This total RNA was used to prepare the first strand of cDNA using reverse transcriptase (GIBCO/BRL) and an oligo dT(17) primer containing an attached short additional nucleotide stretch. The sequence of the primer used is as described in Example 2, 20 under point 2.6. RNase H was subsequently used to remove the RNA from the synthesized DNA/RNA hybrid molecule. PCR was performed using a gene-specific primer (Example 2, point 2.6) and a primer complementary to the attached short additional nucleotide stretch. The thus obtained PCR product was 25 analysed by gel electrophoresis. Fusion constructs were detected by comparing them with the background bands of normal cells of the same individual.

In an additional experiment, a second round of hemi-nested PCR was performed using one internal primer and the 30 primer complementary to the short nucleotide stretch. The sensitivity of the test was thus significantly improved.

EXAMPLE 8

PLAG2 as diagnostic marker.

35 To evaluate the diagnostic potential of the PLAG2 gene, we have first identified on which human chromosome the PLAG2 gene is located and subsequently established its subchromosomal location. Such mapping studies could link the gene to

known tumor-specific chromosome anomalies. Using several different PLAG2 containing YACs and cosmids in FISH analysis, the PLAG2 gene was tentatively mapped to chromosome band 6q24, a chromosome segment implicated in various tumors 5 such as for instance malignant salivary gland tumors.

Malignant salivary gland tumors are a heterogeneous group of tumors comprising at least 17 different entities according to the most recent WHO classification (1991). Because of the large number of tumor types and the wide 10 morphological spectrum that these tumors show they constitute a well recognized diagnostic problem. The most frequent chromosome abnormalities so far observed among malignant salivary gland tumors are deletions or translocations involving 6q. Most of the deletions so far encountered are 15 terminal deletions with breakpoints located at 6q22-q25. However, there are also a few cases on record with interstitial 6q deletions. The minimal common region lost in the majority of all cases is 6q24-qter. The 6q deletions are not restricted to certain types of malignant salivary gland 20 tumors, but seem to occur in all major histologic subtypes, including adenoid cystic carcinoma, adenocarcinoma, undifferentiated carcinoma, mucoepidermoid carcinoma and acinic cell carcinoma. The frequency of 6q rearrangements vary somewhat in different tumor types. In for example adenoid 25 cystic carcinomas clonal 6q deletions or translocations have been found in nearly 50 % of the cases. The fact that the 6q deletions are often the sole karyotypic change, indicate that they are primary cytogenetic events of pathogenetic importance to malignant salivary gland tumors. In addition 30 to 6q deletions a subgroup of adenoid cystic carcinomas are characterized by a t(6;9)(q21-24;p13-23). This is a primary abnormality that is diagnostic for this tumor types. It should also be noted that in addition to malignant salivary gland tumors there are several other tumor types with recurrent 35 deletions of 6q, including e.g. various types of leukemias and lymphomas, malignant melanomas and neuroblastomas.

Involvement of PLAG2 in malignant salivary gland tumors

This example is performed to test whether the different 6q deletions commonly seen in different histologic subtypes of malignant salivary gland tumors involve the PLAG2 gene at 5 6q24.

8.1 Material and methods

FISH analysis using the PLAG2 containing CEPH mega-YACs 798D8 and 921C2 was performed on metaphase chromosomes from 10 two cases of malignant salivary gland tumors, one adenocarcinoma and one adenoid cystic carcinoma, with 6q deletions. The methods for cytogenetic analysis and FISH were the same as described for PLAG1.

15 8.2 Results

Hybridization with both YACs on metaphases from the adenocarcinoma resulted in signals of the same intensity on both chromosome 6 homologs at the expected position. There was no evidence of deletion of PLAG2 signals in this case.

20 In contrast, the adenoid cystic carcinoma contained a subpopulation of cells, which in addition to the signal from the normal chromosome 6 showed either no signal or a weak signal from the 6q-marker chromosome. Both YACs gave a similar hybridization pattern.

25

8.3 Conclusion

Preliminary studies suggest that at least one of the breakpoints in the 6q deletions in malignant salivary gland tumors map in the vicinity of the PLAG2 locus. PLAG2 is 30 therefore a candidate gene for the commonly occurring 6q deletions in malignant salivary gland tumors.

EXAMPLE 10

Animal tumor models involving PLAG1 as tools in in vivo 35 therapeutic drug testing

On the basis of the acquired PLAG1 knowledge, animal tumor models can be developed as tools for in vivo therapeutic drug testing. To achieve this objective (for instance

for pleomorphic adenoma of the salivary gland), two approaches can be used, gene transfer (generation of transgenic animals) on the one hand and gene targeting technology (mimicking in vivo of a specific genetic aberration via 5 homologous recombination in embryonic stem cells (ES cells)) on the other. These technologies allow manipulation of the genetic constitution of complex living systems in specific and pre-designed ways. For extensive technical details, see B. Hogan, R. Beddington, F. Constantini, and E. Lacy; In: 10 Manipulating the mouse embryo, a laboratory manual. Cold Spring Harbor Press, 1994; ISBN 0-87969-384-3.

To aim at the mutation of the PLAG1 gene, specifically in selected cell types and selected moments in time, the recently described Cre/LoxP system can be used (Gu, H. et 15 al. Deletion of a DNA polymerase β gene segment in T cells using cell type-specific gene targeting. Science 265, 103-106, 1994). The Cre enzyme is a recombinase from bacteriophage P1 whose physiological role is to separate phage genomes that become joined to one another during infection. To 20 achieve so, Cre lines up short sequences of phage DNA, called loxP sites and removes the DNA between them, leaving one loxP site behind. This system has now been shown to be effective in mammalian cells in excising at high efficiency chromosomal DNA. Tissue-specific inactivation or mutation of 25 a gene using this system can be obtained via tissue-specific expression of the Cre enzyme.

As an example, the development of animal model systems for pleomorphic adenoma of the salivary glands using a member of the PLAG gene family will be outlined below, such 30 that the models will be instrumental in in vivo testing of therapeutic drugs.

Two approaches will be followed:

- a) in vivo induction of specific genetic aberrations as observed in human patients ((conditional) gene (isogenic) 35 targeting approach); and
- b) introduction of DNA constructs representative for the genetic aberrations observed in patients (gene transfer approach).

DNA constructs to be used in gene transfer will be generated on the basis of observations made in patients suffering from pleomorphic adenomas of the salivary glands as far as structure and expression control are concerned; 5 e.g. PLAG1 fusion genes with various translocation partner genes, especially the preferential translocation partner gene CTNNB1 of chromosome 3, i.e. complete PLAG1 under control of a strong (salivary gland-specific) promoter.

10 **EXAMPLE 11**

The preparation of antibodies against PLAG-encoded proteins

One type of suitable molecules for use in diagnosis and therapy are antibodies directed against the PLAG genes. Two approaches have been followed to develop them. Based upon 15 the nucleotide sequence of the PLAG cDNAs, computer analysis was used to predict the location of antigenic determinants within the proteins. Synthetic peptides containing such determinants were prepared and rabbits were immunized with these. As an alternative approach, cDNA sequences can be 20 expressed in appropriate pro- and eukaryotic expression systems and the polypeptides synthesized in this way can be purified and used for immunization of mice. Cell lines obtained upon transfection or electroporation of constructs of the PLAG genes were helpful in characterizing the antibodies 25 obtained.

For the preparation of rabbit polyclonal antibodies directed against the PLAG1-encoded proteins, use was made of the following three commercially available peptides:

(H-DLSEVRDTQKVPSGKR) 8-Multiple Antigen Peptide

30 (H-FSSTSAYAISIPEKEQPL) 8-MAP

(H-QLPTQTQDLQDP) 8-MAP

obtainable from Research Genetics Inc., Huntsville, AL, USA. The polyclonal antibodies were made according to standard techniques.

35 With respect to PLAG2, suitable antibodies were generated using hybrid proteins as antigens. These contained various portions of the PLAG2 coding region fused in frame to GST (glutathion-S-transferase).

EXAMPLE 12

Inhibition of PLAG1 expression in tumor cells using antisense PLAG1 constructs.

5 1. Introduction

PLAG1 can be used as target in novel therapeutic protocols for tumors in which it is implicated; e.g. pleomorphic adenomas of the salivary glands, uterine leiomyoma, etc. Cancer as a genetic disease is a logical target for gene 10 therapy, either by replacing the missing or inactivated gene or by suppressing the activity of an unwanted gene. The high affinity of short DNA sequences for their target mRNA has indicated that antisense oligonucleotides constitute valuable reagents to specifically suppress the production of gene 15 products, both in vitro and in vivo. Applications have been documented in various biomedical research areas, such as for instance cancer research, virology, and cardiovascular research. The in vitro activity, toxicity, and pharmacokinetic data of antisense oligonucleotides are encouraging 20 and in vivo animal experiments demonstrating suppression of neointimal formation seem very promising. Also in the field of cancer research promising results have been obtained. However, potential nonspecific effects of antisense oligonucleotides should be carefully considered when antisense 25 agents are used to define biological functions of specific genes.

Since expression of the PLAG1 gene is frequently activated or strongly elevated in a wide variety of tumors and tumor cell lines, derived from tumors (rhabdomyosarcoma, 30 uterine leiomyosarcoma, uterine leiomyoma, malignant salivary gland tumors) as well as leukemias and lymphomas, it was speculated that the PLAG1-encoded protein might play a key role in transformation of cells. This example shows that expression of the PLAG1 gene can be strongly reduced by 35 expressing antisense PLAG1 sequences and that reduction of PLAG1 levels in tumor cells results in reversion of the transformed phenotype. Thus the expression or administration

of antisense molecules can be successfully applied therapeutically.

2. Materials and methods

5 2.1 Tumor cell lines

Tumor cell lines were generated from primary tumors as described by Kazmierczak *et al.*, *Genes Chromosom. Cancer* 5:35-39, 1992.

10 2.2 Culturing tumor cells

Tumor cells were propagated in TC199 culture medium with Earle's salts, supplemented with 20% fetal bovine serum (GIBCO), 200 IU/ml penicillin, and 200 microgram/ml streptomycin.

15

2.3 Transfection assay

Transfections were performed using various protocols, namely:

1. The calcium phosphate precipitation procedure of Graham and Van der Eb ("A new technique for the assay of the infectivity of human adenovirus." *Virology* 52: 456-467, 1973.).
2. Lipofection: Transfections were carried out using liposome-mediated DNA transfer (lipofectamine, GIBCO/BRL) according to the guidelines of the manufacturer.

25

2.4 Antisense constructs

Sense and antisense constructs of the PLAG1 gene were obtained by inserting human PLAG1 cDNA sequences in both the sense and antisense orientation in expression vectors under transcriptional control of various promoter contexts, e.g. the long terminal repeat of Moloney murine leukemia virus, a CMV promoter, or the early promoter of SV40. For example, the CMV/PLAG1 plasmid was constructed by cloning a human PLAG1 cDNA fragment containing all coding sequences of human PLAG1 in pRC/CMV (Invitrogen) allowing expression under control of the human cytomegalovirus early promoter and enhancer, and selection for G418 resistance.

3. Results3.1 Inhibition of PLAG1 expression

Inhibition of PLAG1 expression was observed in tumor cells after induction of antisense PLAG1 expression in these 5 tumor cells. Immunoprecipitation and Western blot analysis indicated a strong reduction of PLAG1 protein levels in the cells expressing antisense PLAG1 sequences. Therefore, this approach can be used therapeutically in tumors with involvement with PLAG1.

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CLAIMS

1. Gene being in an isolated form and implicated in tumorigenesis having the nucleotide sequence of any one of the strands of any one of the members of the PLAG subfamily of zinc finger protein genes or CTNNB1 genes, including 5 modified versions thereof.
2. Tumorigenesis gene (T-gene) as claimed in claim 1 having at least homology with the zinc finger domains that are typical for the PLAG1 gene the nucleotide sequence of which is depicted in figure 4A, or the 10 complementary strand thereof, including modified or elongated versions of both strands.
3. T-gene as claimed in claim 1 or 2 having essentially the nucleotide sequence of the PLAG1 gene as depicted in figure 4A, or the complementary strand thereof, including 15 modified or elongated versions of both strands.
4. T-gene as claimed in claim 1 or 2 having essentially the nucleotide sequence of the PLAG2 gene as depicted in figure 8A, or the complementary strand thereof, including modified or elongated versions of both strands.
- 20 5. T-gene as claimed in claim 1 having essentially the nucleotide sequence of the CTNNB1 gene as depicted in figure 9, or the complementary strand thereof, including modified or elongated versions of both strands.
- 25 6. T-gene as claimed in claims 1-5 for use as a starting point for designing suitable expression-modulating compounds or techniques for the treatment of non-physiological proliferation phenomena in human or animal.
- 30 7. T-gene as claimed in any one of the claims 1-6 for use as a starting point for designing suitable nucleotide probes for (clinically/medically) diagnosing cells having a non-physiological proliferative capacity as compared to wildtype cells.
- 35 8. T-gene as claimed in claims 1-7, which gene is implicated in tumors selected from rhabdomyosarcoma, uterine leiomyosarcoma, uterine leiomyoma, malignant salivary gland tumors, leukemias and lymphomas.

9. Protein encoded by a T-gene as claimed in claims 1-8 for use as a starting point for preparing suitable antibodies for (clinically/medically) diagnosing cells having a non-physiological proliferative capacity as compared to wildtype cells.

10. Derivatives of the T-gene as claimed in claim 1-8 for use in diagnosis and the preparation of therapeutical compositions, wherein the derivatives are selected from the group consisting of sense and anti-sense cDNA or 10 fragments thereof, transcripts of the gene or practically usable fragments thereof, fragments of the gene or its complementary strand, proteins encoded by the gene or fragments thereof, antibodies directed to the gene, the cDNA, the transcript, the protein or the fragments thereof, as 15 well as antibody fragments.

11. In situ diagnostic method for diagnosing interphase and/or metaphase cells having a non-physiological proliferative capacity, comprising at least some of the following steps:

20 a) designing a set of nucleotide probes based on the information obtainable from the nucleotide sequence of the T-gene as claimed in claim 1-5, wherein at least one of the probes is hybridisable to a region of the aberrant gene substantially mapping at the same locus as a corresponding 25 region of the wildtype gene and/or the same or another probe is hybridisable to a region of the aberrant gene mapping at a different locus than a corresponding region of the wildtype gene;

30 b) incubating one or more interphase or metaphase chromosomes or interphase or metaphase cells having a non-physiological proliferative capacity, with the probe(s) under hybridising conditions; and

c) visualising the hybridisation between the probe(s) and the gene.

35 12. Method of diagnosing cells having a non-physiological proliferative capacity, comprising at least some of the following steps:

- a) taking a biopsy of a tumor to obtain cells to be diagnosed;
- b) isolating a suitable T-gene-related macromolecule therefrom;
- 5 c) analysing the macromolecule thus obtained by comparison with a wildtype reference molecule preferably from the same individual.

13. Method as claimed in claim 12, comprising at least some of the following steps:

- 10 a) taking a biopsy of a tumor to obtain cells to be diagnosed;
- b) extracting total RNA thereof;
- c) preparing at least one first strand cDNA of the mRNA species in the total RNA extract, which cDNA comprises a suitable tail;
- 15 d) performing a PCR and/or RT-PCR using a PLAG gene specific primer and a tail-specific and/or partner-specif/nested primer in order to amplify PLAG gene specific cDNA's;

20 e) separating the PCR products on a gel to obtain a pattern of bands;

f) evaluating the presence of aberrant bands by comparison to wildtype bands, preferably originating from the same individual.

25 14. Method as claimed in claim 12, comprising at least some of the following steps:

- a) taking a biopsy of a tumor to obtain cells to be diagnosed;
- b) isolating total protein therefrom;
- 30 c) separating the total protein on a gel to obtain essentially individual bands and optionally trnasferring the bands to a Western blot;
- d) hybridising the bands thus obtained with anti-bodies directed against a part of the protein encoded by the
- 35 remaining part of the T-gene and against a part of the protein encoded by the substitution part of the T-gene;
- e) visualising the antigen-antibody reactions and establishing the presence of aberrant bands by comparison

with bands from wildtype proteins, preferably originating from the same individual.

15. Method as claimed in claim 12, comprising at least some of the following steps:

- 5 a) taking a biopsy of a tumor to obtain cells to be diagnosed;
- b) isolating total DNA therefrom;
- c) digesting the DNA with one or more so-called "rare cutter" restriction enzymes;
- 10 d) separating the digest thus prepared on a gel to obtain a separation pattern;
- e) optionally transferring the separation pattern to a Southern blot;
- f) hybridising the separation pattern in the gel
- 15 or on the blot with one or more informative probes under hybridising conditions;
- g) visualising the hybridisations and establishing the presence of aberrant bands by comparison to wildtype bands, preferably originating from the same individual.

20 16. Method as claimed in any one of the claims 10-15, wherein the cells having a non-physiological proliferative capacity are selected from the group consisting of the tumors pleomorphic adenomas of the salivary gland, lipoblastomas, uterine leiomyomas, and other 25 benign tumors as well as various malignant tumors, including but not limited to sarcomas (e.g. rhabdomyosarcoma, malignant salivary gland tumors) and leukemias and lymphomas.

17. Anti-sense molecules of a T-gene as claimed in 30 claims 1-8 for use in the treatment of diseases involving cells having a non-physiological proliferative capacity by modulating the expression of the gene.

18. Expression inhibitors of the T-gene as claimed in claims 1-8 for use in the treatment of diseases involving 35 cells having a non-physiological proliferative capacity.

19. Diagnostic kit for performing the method as claimed in claim 11, comprising a suitable set of labeled nucleotide probes.

20. Diagnostic kit for performing the method as claimed in claim 12, comprising a suitable set of labeled probes.

21. Diagnostic kit for performing the method as claimed in claim 13, comprising a suitable set of labeled T-gene specific and tail specific PCR primers.

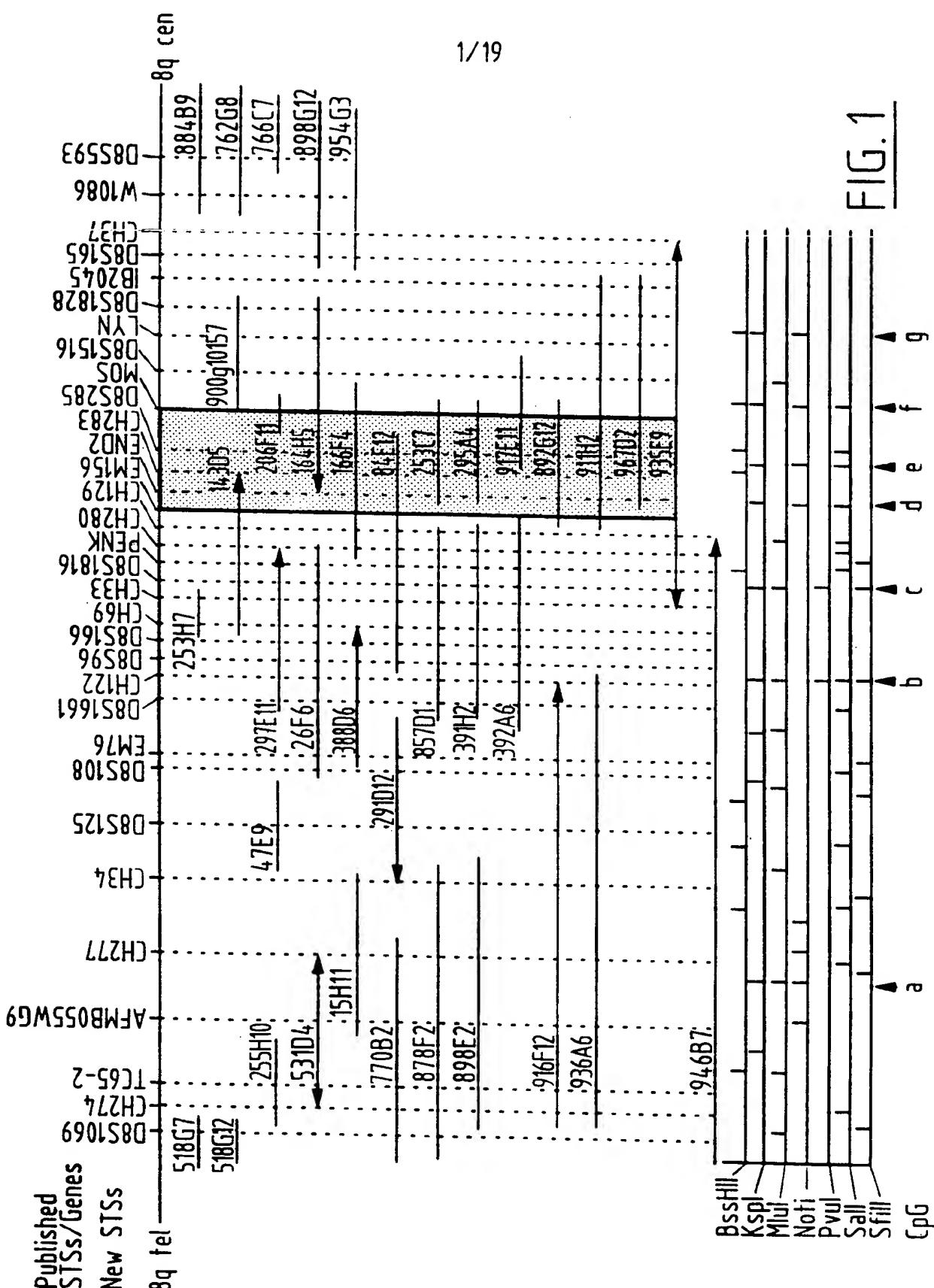
22. Diagnostic kit for performing the method as claimed in claim 15, comprising a suitable set of labeled probes, and suitable rare cutting restriction enzymes.

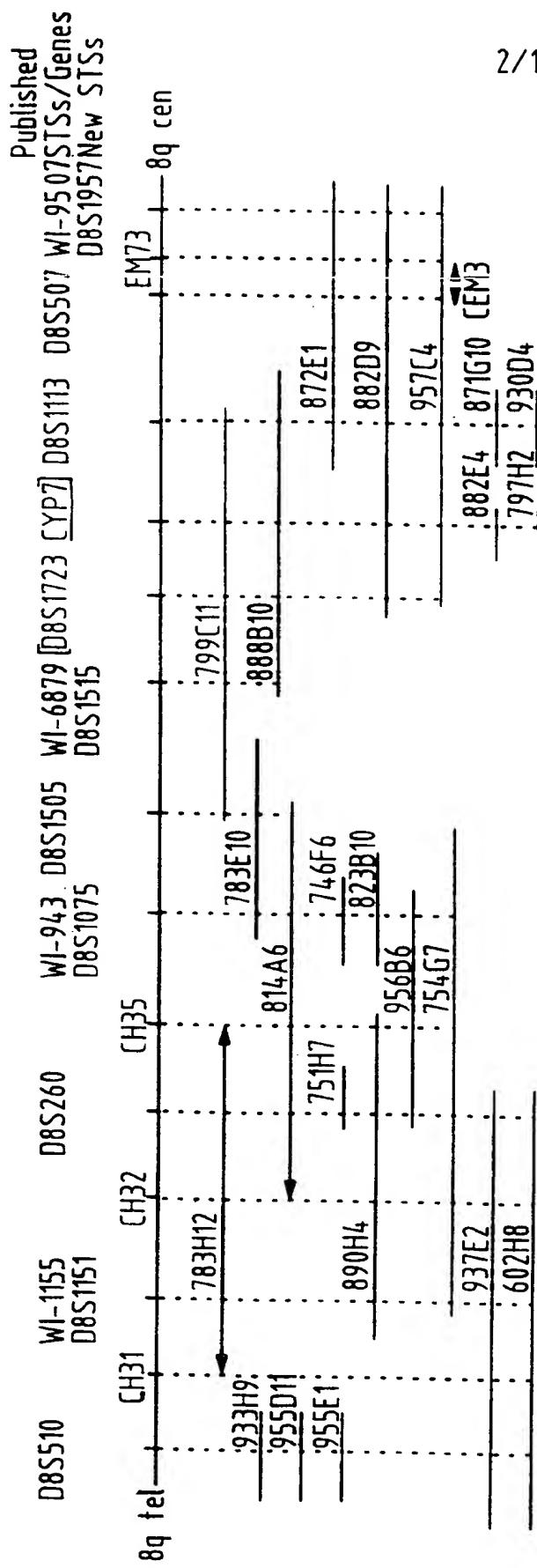
10 23. Method for isolating other T-gene based on the existence of a fusion gene, fusion transcript or fusion protein in a tumor cell by using at least a part of a T-gene for designing molecular tools (probes, primers etc.).

24. T-gene obtainable by the method of claim 23.

15 25. T-gene as claimed in claim 24 for use in diagnostic or therapeutic methods.

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FIG. 2

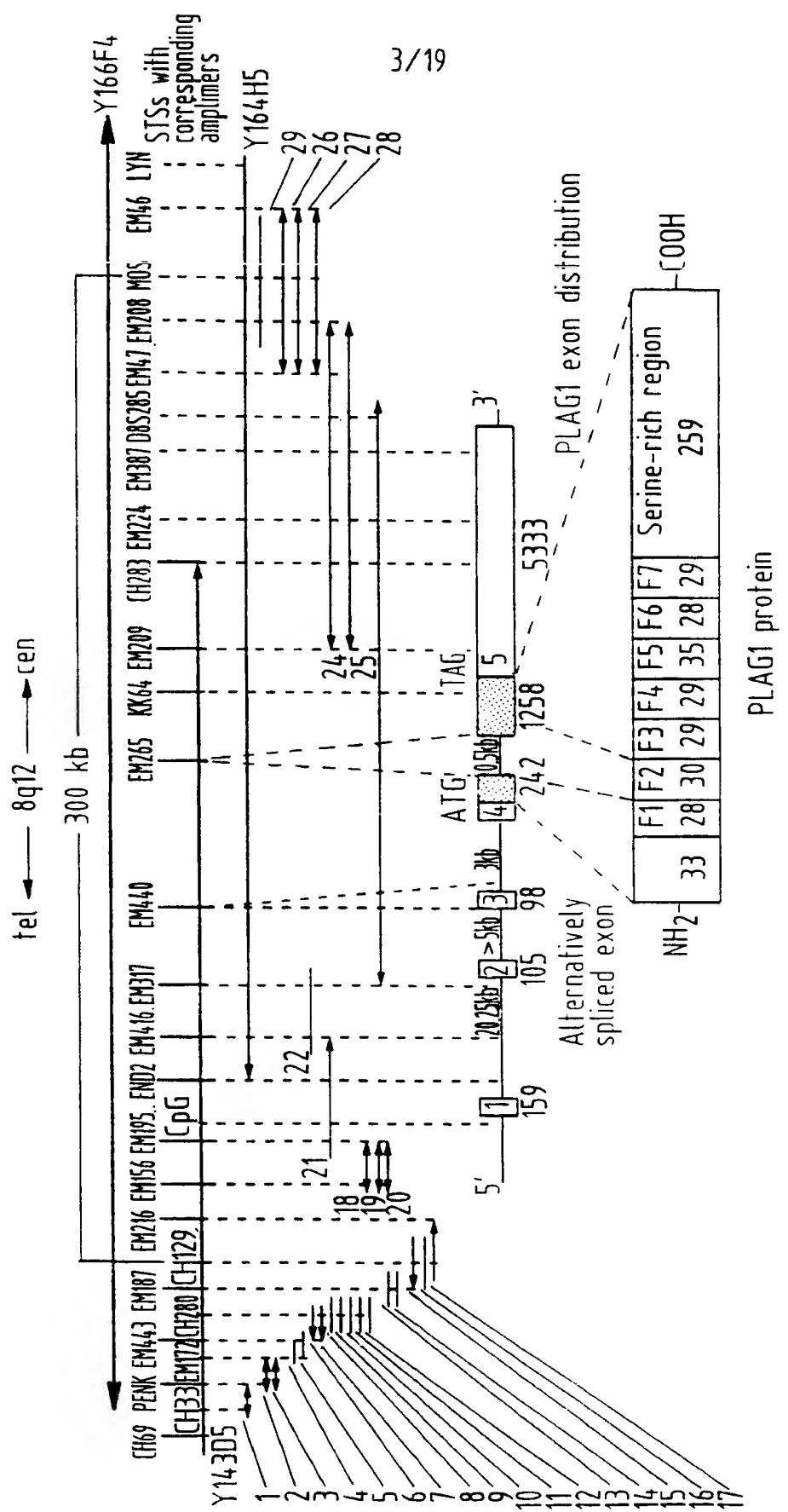
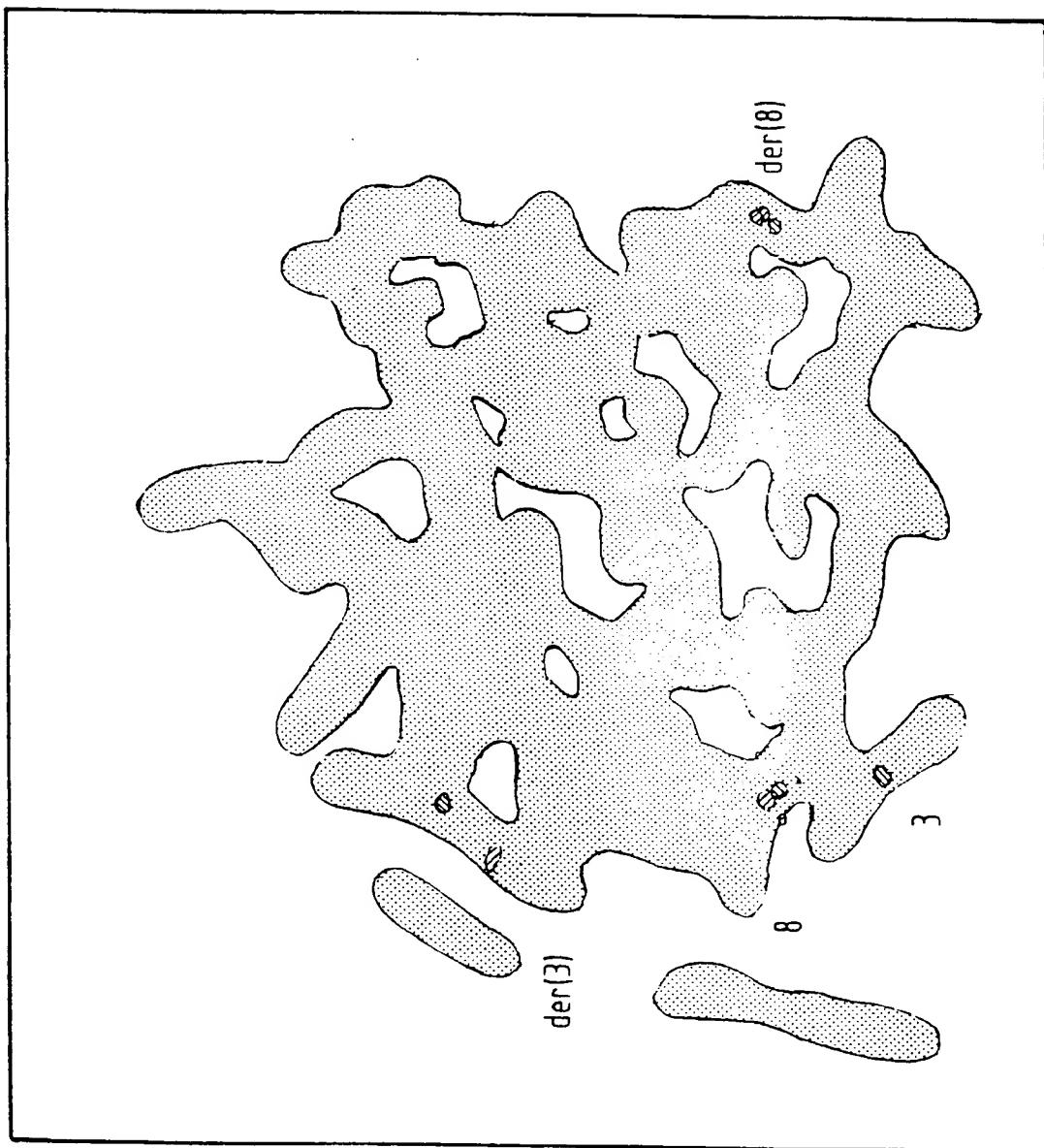


FIG. 3A

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FIG. 3B

PLAG1 cDNA sequence

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FIG. 4A

GGCAGCGCAT	ACACTACAAT	GGCTGCTGGA	AAGAGGCGTA	AGGAAACAAT	50
TTCCAGGCC	GCCGCGTCCA	GCCCGAAATA	TGAGAAAAAA	ATTATTAGAA	100
ATTCCGCGGG	CGGTGTAGAG	GCGGCGGACG	GGCCGGAGGG	AGGATGTTAA	150
AGCCCCGCGG	TTGCCTCTTG	GTGCTGCCTT	GGCCGTATT	GGCACCCAGA	200
ATGCTTCATT	CTGTGACGCT	CTATTAATAA	GGTGCCTTG	CTAGAGTTTG	250
GAGCAGGGCC	TCAGATTGGC	CAAAATGGGA	AGGATTGGAT	TCCACTCTCT	300
TCCACGAAGA	GTCAATGGGA	CTGGCTAAGA	TCAAAGTCTG	AGGCTTTTC	350
CATCAGTAAT	CAGTCCCTTT	TTGCTTTCTT	TTACGACCAC	ATGAAACTTG	400
AGAAGCCACC	TAAAGCTATA	TCATTTAGTG	GAGTTGGGCA	GTTCCAAGT	450
GTCCAACAAAG	AAGGCCTGGT	TTAGGCTGCG	ATGCCACTG	TCATTCTGG	500
TGATTTGTCA	GAAGTAAGAG	ATACCCAGAA	AGTCCCTTC	GGGAAACGTA	550
AGCGTGGTGA	AACCAAACCA	AGAAAAAAACT	TTCCTTGCCA	ACTGTGTGAC	600
AAGGCCTTTA	ACAGTGTGTA	GAAATTAAAG	GTTCACTCCT	ACTCTCACAC	650
AGGAGAGAGG	CCCTACAAGT	GCATACAACA	AGACTGCACC	AAGGCCTTTG	700
TTTCTAAGTA	CAAATTACAA	AGGCACATGG	CTACTCATT	TCCTGAGAAA	750
ACCCACAAGT	GTAATTATTG	TGAGAAAATG	TTTCACCGGA	AAGATCATCT	800
GAAGAATCAC	CTCCATACAC	ACGACCCCTAA	CAAAGAGACG	TTTAAGTGC	850
AAGAATGTGG	CAAGAACTAC	AATACCAAGC	TTGGATTAA	ACGTCACTTG	900
GCCTTGCATG	CCGCAACAAAG	TGGTGCACCTC	ACCTGTAAGG	TATGTTGCA	950
AACTTTGAA	AGCACGGGAG	TGCTTCTGGA	GCACCTTAAA	TCTCATGCAG	1000
GCAAGTCGTC	TGGTGGGGTT	AAAGAAAAAA	AGCACCAAGT	CGAACATTGT	1050
GATCGCCGGT	TCTACACCCG	AAAGGATGTC	CGGAGACACA	TGGTGGTGCA	1100
CACTGGAAGA	AAGGACTTCC	TCTGTCAGTA	TTGTGCACAG	AGATTGAGGC	1050
GAAAGGATCA	CCTGACTCGA	CATATGAAGA	AGAGTCACAA	TCAAGAGCTT	1200
CTGAAGGTCA	AAACAGAAC	AGTGGATTTC	CTTGACCCAT	TTACCTGCAA	1250
TGTGTCGTG	CCTATAAAAG	ACGAGCTCCT	TCCGGTGTAT	TCCTTACCTT	1300
CCAGTGAACT	GTATCAAAG	CCATTACAA	ACACTTTGCA	GTTAACACCTC	1350
TACAACACTC	CATTTCAGTC	CATGCAGAGC	TCGGGATCTG	CCCACCAAAT	1400
GATCACAAC	TTACCTTTGG	GAATGACATG	CCCAATAGAT	ATGGACACTG	1450
TTCATCCCTC	TCACCACCTT	TCTTCAAAT	ATCCGTTCA	TTCTACCTCA	1500
TATGCAATT	CTATTCTG	AAAAGAACAG	CCATTAAAGG	GGGAAATTGA	1550
GAGTTACCTG	ATGGAGTTAC	AAGGTGGCGT	GCCCTCTTCA	TCCCAAGATT	1600
CTCAAGCATC	GTCATCATCT	AAGCTAGGGT	TGGATCCTCA	GATTGGGTCC	1650
CTAGATGATG	GTGCAGGAGA	CCTCTCCCTA	TCCAAAAGCT	CTATCTCCAT	1700
CAGTGCACCC	CTAAACACAC	CAGCATTGGA	TTTTTCTCAG	TTGTTTAATT	1750
TCATACCTT	AAATGGTCT	CCCTATAATC	CTCTATCAGT	GGGGAGGCCTT	1800
GGAATGAGCT	ATTCCCAGGA	AGAAGCACAT	TCTTCTGTT	CCCAGCTCCC	1850
CACACAAACA	CAGGATCTC	AGGATCCTGC	AAACACTATA	GGGCTTGGGT	1900
CTCTGCAC	ACTGTCAGCA	GCTTCAACCA	GCAGTTAAC	CACAAGTACC	1950
ACCCCTCCAC	GTTCATCATC	AGCTTTCA	TAGGATTCTG	GGACATGGAT	2000
TCATTACAGA	AATGTATGTG	AGCTGTG	CTAGATGACC	ATTTTTATTT	2050
TAGTGCCTAC	TTTAAACAG	TATAAAAATT	TCTGCTTTG	TATAATACAA	2100
ATTTTCATTA	AGCCAGTATA	AAATAGAAAC	AGCTTTAA	ACTGAGCTTT	2150
GGAACCATT	GTGTTCA	AAGTTACCT	GGGTATTTG	TCCTGATTCA	2200
CTGCCAATTG	TCACATT	AGACTTTTT	TTTTCCATA	TAGGAAAGCC	2250
ATTATTAGTA	GTAAACTTT	ACAAATCCC	TTTCAAATT	ACTTTAGAT	2300
CTTAAAATT	TCATTTTG	CTAATAACAG	TGGCTCTACC	TTTGACATC	2350
TGGCTCATTA	AAAAATTAG	CAATAGAATG	TAAATTGTAT	AAAAAGTTG	2400
TGAATAACTC	AAGGGTTAA	ATTTCCTAC	AGCTTCTAA	ATGGATTAAT	2450
AATCAAGTGC	TTCAAATGAA	TAAAGAGTCC	AGTTCGGAA	GATAATAAAT	2500
GTTGTTAGA	TACACCATAA	TTTCAGATCA	GTATATTCTG	AAGACTCTCT	2550
GTTGTCGGC	TAATATTTT	GCCATCTTAA	TTATGAGCCT	TTAAGGAAAA	2600
CAAACCTAA	ACACAAAGCA	TCAGTATT	TAGCAAAAG	AGACTCTGTT	2650

6/19 FIG. 4A (continued)

AGGTGACATG	GCATTTCGTG	TCACCTAATA	GTTGGCCCTA	AATTAGTACA	2700
CAGGATATTT	TGTCGTGTT	CATCCTTCTT	AAACATGCTAT	CTTTTCATT	2750
AATAATAGTA	ATAGTGTATG	GCATTGGGGT	CTTCAGAGTC	GATATATAGG	2800
TAGATCTCTT	TAGTCTTTTC	CACCTTCAC	ATCCAAGGGG	TGGGTCAAGT	2850
GCAGCCAGCA	ATTTATTTC	ATTGTTGGCC	CACGGTTAGT	CCATAATCTA	2900
GAGCCATTGT	GGAAC TGCA G	CCATGAGGTG	TGTTTATCCC	ACAGTGGATT	2950
GACTCAGCCT	CTGTGGGTGA	CAGACTTCTA	AGCAGGAAGA	TAGACGTGAA	3000
GCACATGGTT	ACATTGGGA	ACTTGTGTA	GGATCATGGC	CCCTGTAGCC	3050
AGGGTTAAAAA	ACTGGACTT	TTAGAAGTAA	AGTAAAAGCA	TAKCGCTTAT	3100
ATCATTCTT	GCTGAATTG	ATATGTTTT	CTTTCCTTA	AGAATCAAAA	3150
GCAGAAAACA	AAAACAACAG	TCCTACTCCG	ATGTTATCTT	TCTGATTCAA	3200
TGTGAATCCA	TCTTCCTTG	CAATATTG	GATGGAGAAT	TTGAAGTTAA	3250
ATGCATTAGA	AAACTACCTG	ATGAAC TACC	ACAAAAGTTT	AAGTGACTAG	3300
AAATATATAC	AGTAAAATCC	CACTTCATG	CATCTCTGGG	AAATGATAGG	3350
AGTATTGCAA	ATAAGTTGAG	TTTGTAGAGG	GTAACAAAGT	AAAGTAAAAC	3400
AAACCTATCT	TGGTTAACAT	GAAAATAACA	ATTGAGAATA	TATTATATTC	3450
ACTGAATAAT	TATAGGCTT	TCCTCACATT	AGACAACCAA	CATAATCTTC	3500
TTAAAGGTCT	ATTAAATATA	TTTTCTAAG	GGTCAGTTGG	GACATTAACC	3550
TAAGGAAACAT	ATCTATTAAAG	CACTTGTAA	CACCTTATT	TAGGACCCTT	3600
TCCGTTGGGG	ATGGGGCAA	GGGTGGGAGG	TTTTAGAAG	AGTATATATC	3650
TCTTTAAAAA	AAAACAGAAA	GAAAATATT	TCTGAGCACT	CATTAGCCCT	3700
ATATGAAAC	TTCTTCCTT	TTTGTAGGGC	CAGTTATCAC	TGCAGATTGC	3750
AATGTTTACC	AAGAATTCT	AAAAATGAGT	GCAGATTACT	GAATATAATA	3800
CATTATTAA	AAATTTGGG	AGTAGTATAA	TTTGTGAGA	AATGAAATT	3850
GTAATAATGT	AAATGGGGG	CTTCAATATA	TATATATAAT	ACACACACAC	3900
ACACACATGC	ACACATACCG	CACTTCATAG	AATCAAAGTT	GCTCTCTGAA	3950
GGAGCTTGG	CTCCTGATAT	TTTATCATGC	TCCTATATT	TTTTAATCCT	4000
TGGAGCAGTA	GTTTTTATAC	TTATGTATT	AAATTTTATT	ATGAAAATT	4050
ACATTATTAA	AAAAAGTGT	TTCCAAAGGC	ATTAATTTA	TATATGTTAA	4100
TAAGGAAGTA	CATTTTAAA	TTTTCAAAAC	TGCTCCTAGC	TTTGATTAG	4150
GAGAATATT	TTTCTGAAAG	TAGGCTTT	GCTCTGCTTC	ATTACTGCTT	4200
CCTTAGTT	CTATGAAACA	GATTGCTTAC	CTAAATCTT	AGTTGAATGA	4250
TTAGTGTCA	ATATTGCTT	AATCACCATA	AAAAAGGAAA	AAAATTGGTG	4300
ACAGAGCACA	AATAGAAAAC	CTATTTTAA	ATAGAAATCA	CAAATAGCAA	4350
GTGTGGAAGC	ACTACTTAT	TCTGTTAAA	ATGTACTTAA	GAAGTCATCA	4400
AATTAGTGA	CTGAGACATT	GGCCTTAGTA	GGCTGTATT	ACTGCTAATT	4450
AAAAAAAAGG	AGTACCAAGGA	TTTATTAAGT	AAAGCATTT	GGAAATGGGG	4500
AATAGCGCCA	TATATGTATG	TATGTGTATG	TGTGTGTGT	GTGTGTGTAT	4550
ATATACACAC	ACACATACAT	ACTTAAATCT	TGCCCTGCAT	GAAATTCAA	4600
TACATGGAGG	CACATCTTC	GGCACCAGT	GTAAAATT	TGGAGTCTTA	4650
ATTTCATGT	GTACACCTCT	TTGCCTGTTC	CCACCCCCAG	ACTTGAAATA	4700
ACACTTCAGA	GTAAGAGGG	ATTCAAGCTAA	TTTGTTTTA	AAATTGACTG	4750
TAGTGGTCAC	TAACCCCTT	TTGAGAGAAT	TTCTATTAAA	GATGAGGCAG	4800
ACTCGCTTAT	TTGAATTGCA	CAATGTTCTA	ACAAGGATGT	AACACAGAAT	4850
TGGCTTTTT	TTCCCTAGAA	AAAGATTGTT	TGTTTCTATG	TCAACTAGAT	4900
ATGATTAAAAA	ATAAGTATTG	CCAATGCTGT	TTTCATTCTC	TAGTGGCCAG	4950
AATCATTATC	CTTGAAATT	CTGGTAGTGC	CTTAGCTTGG	TTAAAAAAA	4500
AAAAAAAAAA	AAAAAAAAG	GGATTAACAT	TAATAAAAAG	TAGTTAGAA	4550
TTTGGGCCTC	AGACAAGATA	TTGAACCTCA	TTCACTTCA	CTTCCACATG	5100
TATGTACAAG	TTAGGTCA	AAACACGGAA	GTTGAGTGT	GAAGGATCTT	5150
GGCACTGTAA	GCAATGCTAT	CCATTGATGT	ATACAAGTAC	CTTTATAGTT	5200
ATCGATCACT	GTAAAACCT	TCATTTAAA	ATCCTATTAC	CAAGTTCACT	5250
TTTTTAAAC	TTCAATTGTC	CTGGCTGATT	ATGCATCACT	CTGTGTGCAA	5300
CTTTTTATT	TCATTTAGTG	TTTCTTCAA	GCTGTGTATT	TTTGCCATT	5350
TGTTGCTTGT	GCTTTATT	TCTTAGTCAT	TTGTGGAATA	TAGTGATATA	5400

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FIG. 4A (continued)

TTGTGTTAAT	TTGGACAGTA	GCGGTTTTA	AAAACCATAT	ACTGACTGAA	5450
ACATGAGCCA	GAGCCGATTG	CTTATTAAAG	CTAATAATGA	ATGTTAAAGA	5500
GTACATATT	TCAGGATCGT	TCATCTAGTG	AGCAATAACAC	ATATTATAGG	5550
CCAATATT	TTTAAAAAAAT	AGAGCTTGGT	CAACCTCTAT	ACTACACATA	5600
TTACAAGATA	TAGCACTTTC	AAAATGAATC	TAAACCTTTA	CAGAAACTTT	5650
CTTATAGGTT	ATGCCTTTA	TTTAAGACT	TATTATAATT	CAAGTGCCAT	5700
TAGATGATAT	ATATGTAGGC	CTTGATATA	TAATGCTTGT	TGTACAAAAAA	5750
TGGTAGATGG	TATTTTAAAC	AGGTACATTT	TTACAGTGT	TTCTTATCAA	5800
TTTGCTATAT	TGCACAGAAAT	CAGTGTGTGT	CTTTTCATAA	GGTTTTACAA	5850
TGGTTTATTT	TTTTACAAGG	TTTACGTGTC	TCAAAGCACA	CTGTCTTCCC	5900
AGTACGTAAG	TTAAAAAATA	CCAGTTCACCC	CAAGTTGCTT	CTAGCCTACT	5950
GAGATCCATG	TGACATTGGA	GGAGATCTT	TAATGTTTA	GTATTCGTCA	6000
TTAGCAATGG	CTGGCTGTTA	GTTCCTGGTAA	ATGTGTGCCT	AAGTTGAATT	6050
TGTCTTGT	TTCTCACACT	GTGTCAGCAG	CCATGTCTAC	AACACAGATA	6100
AGTCTGTTGT	GATCACATAG	ATCTACATAA	GTGTCAGT	TTTGTGCTAA	6150
AAACCCATAG	GGAGCTCCTT	TGGGATCATA	GAAAAGAAGA	TCATGCAACC	6200
AGCATTGGTG	AAGGCACACT	CAGATTGCAC	TTAGGGCCTT	TCTATGATGT	6250
TGTCAACCT	CTGAGGATGG	AAGGCAGTGT	CTTTTGATGT	TATCTAGCCT	6300
AGAAATGACA	CAGAACTATT	GCTAATGTAT	AAAACACTTC	ATTATATAAG	6350
CTTCAGTGGT	ACAGATGAAC	CAGAAATGAAT	GTTCATCTTC	TCAGAAACAC	6400
TCCTTCAATA	TTATATTGGA	TCATGCTGCT	AATGTAACCTT	GGGCTACAAC	6450
TCTTCATGGT	GCTACAAACT	TCTCTGTCTC	ATTCACTCGT	ATTTTTTAT	6500
CCATAGAAAA	AGGACTACAT	TAGGTGTAAA	AGTGTACAAT	ATATTTTAT	6550
ACTGTGACTT	AATTTGTCAT	TAACAAACTT	TTACACCACC	ACAATGTATT	6600
CATGTGCACT	TGCAAAAGGA	GATCTCGGAC	ATGCAAATGT	TACCAGAACAA	6650
AACCCAGCTT	TTGTCCACAA	GGTGAATGTA	ACTCAGAATG	GAAAGTGGGC	6700
TTTATAATAG	GGTGTGGAGT	GAAGAACATG	CTGTATGTTA	CTAACAGCCC	6750
TTTGAATT	ACAAAAAACTG	GGAATCCATT	AGGAAACGGG	TTGCATCATA	6800
CCTGAACATA	AGCTGGACTG	CTGAAATTGT	ATTTTTAGCT	AATGAAAAAG	6850
TGTTTGGACT	AGTACTCTAA	AAATGTTCTA	ATGATAAAAGT	TTTGAGTC	6900
AATAGAAAAG	AAAAAAATCT	GCATTCCAGG	CCGAATTG	TATATTTTAA	6950
TTGCATTAA	AATTGCTATT	CTGTAATATT	GGGAAATCAA	GTGGCTTATC	7000
ATGTATATCG	TGTACTTAAA	ATGTATTCA	AAACTACTGT	TGTATTTGTA	7050
TAAAATATAG	ACAAAGATCA	TATTTTTGT	GTGTGTATAA	GCTCTGTAAA	7100
ATAGCAATCA	CATTATGAAG	CTGCAGTGT	ACTACATTTT	AAACATTCA	7150
ATCCAAAGAA	GCAGACTATT	TATTGTCCAT	ATACCAGATT	TAAAATATTA	7200
ATTTGCTGCT	AATTAAATAA	TAGTACTGCA	GCTCTTGTG	GCCTACAGTG	7250
TTATGTTGC	TGTAAGAATA	AGATATGTGA	ATTCCACAAA	ATATATGAAT	7300
AAAATCTCGT	GCC	7313			

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PLAG1	Finger 1	FPC..QLCDKAFNSVEKLKVHSYS.HTGERP
PLAG1	Finger 2	YKCJQQDCTKAFVSKYKLQRHMAT.HSPEKT
PLAG1	Finger 3	HKC..NYCEKMFRKDHLKNHLHT.HDPNKET
PLAG1	Finger 4	FKCEE..CGKNYNTKLGFKRHLAL.HAATSGD
PLAG1	Finger 5	LTC..KVCLQTTESTGVILLEHLKS.HAGKSSGGVKEKK
PLAG1	Finger 6	HQCEH..CDRRFYTRKDVRHHMVV.HTGRKD
PLAG1	Finger 7	FLC..QYCAQRFGRKDHLTRHMKSHNQELL
PLAG1	Consensus	..C....C...F.....L..H....H.....
C2H2	Consensus	FxCxxxxCxxxFxxxxxLxxHxxxxHxxxxx Y

FIG. 4B

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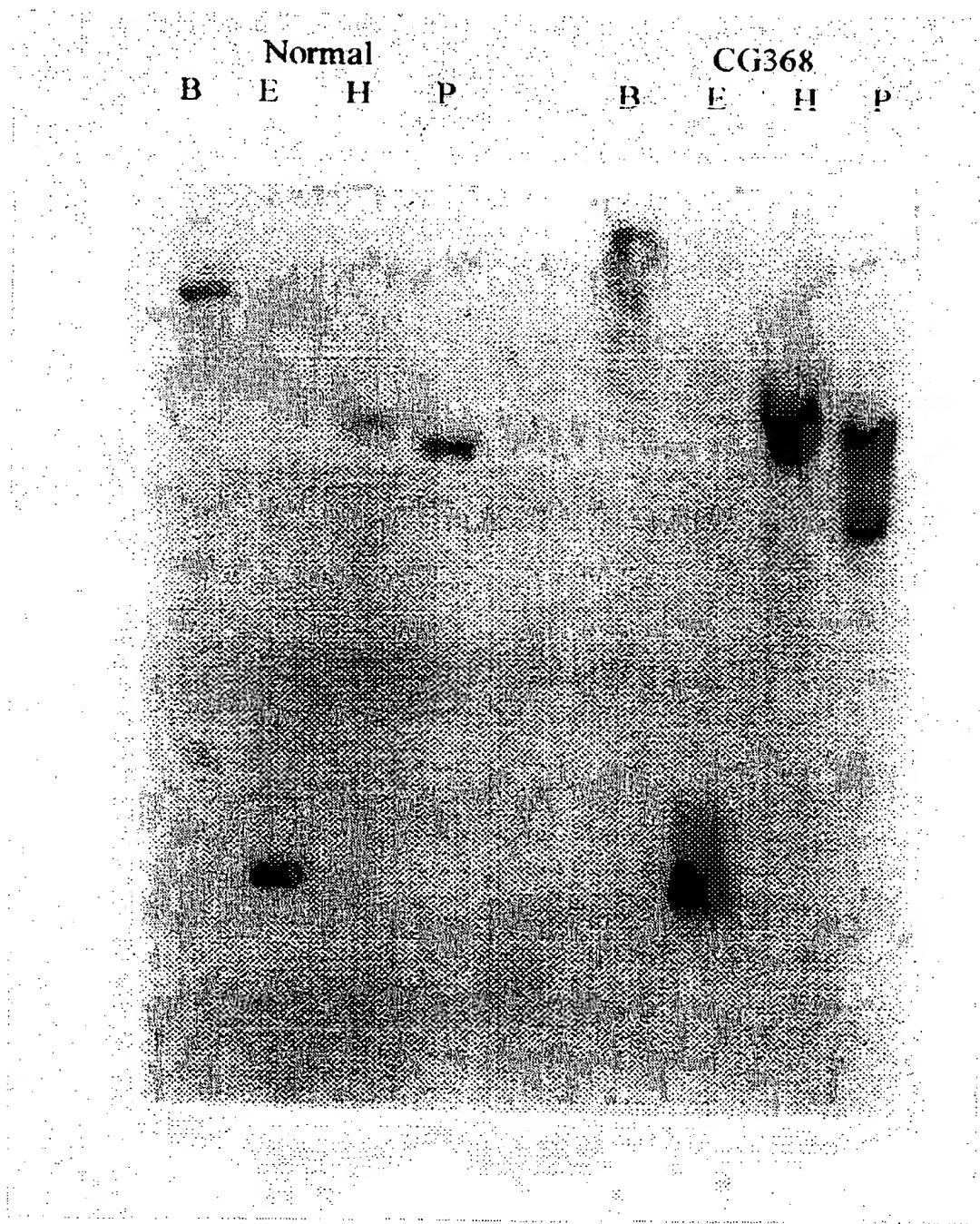
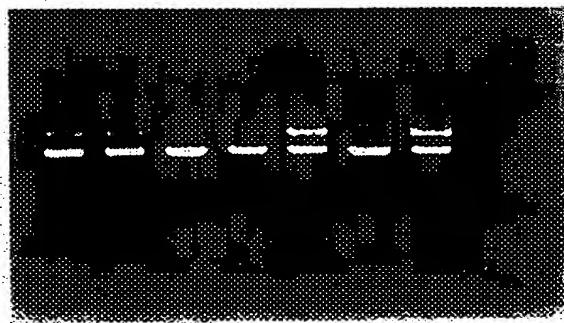


FIG. 5

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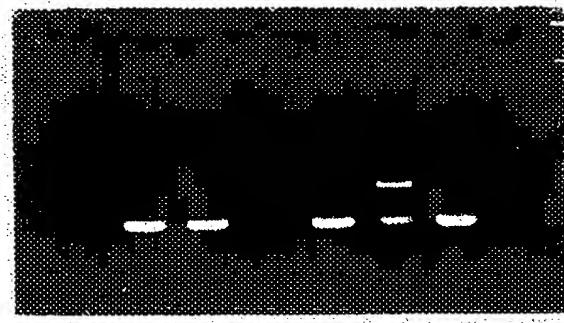
CG3613
CG588
CG644
CG682
CG752
CG753
T9587
CG580

1 2 3 4 5 6 7 8



↑↑
A B

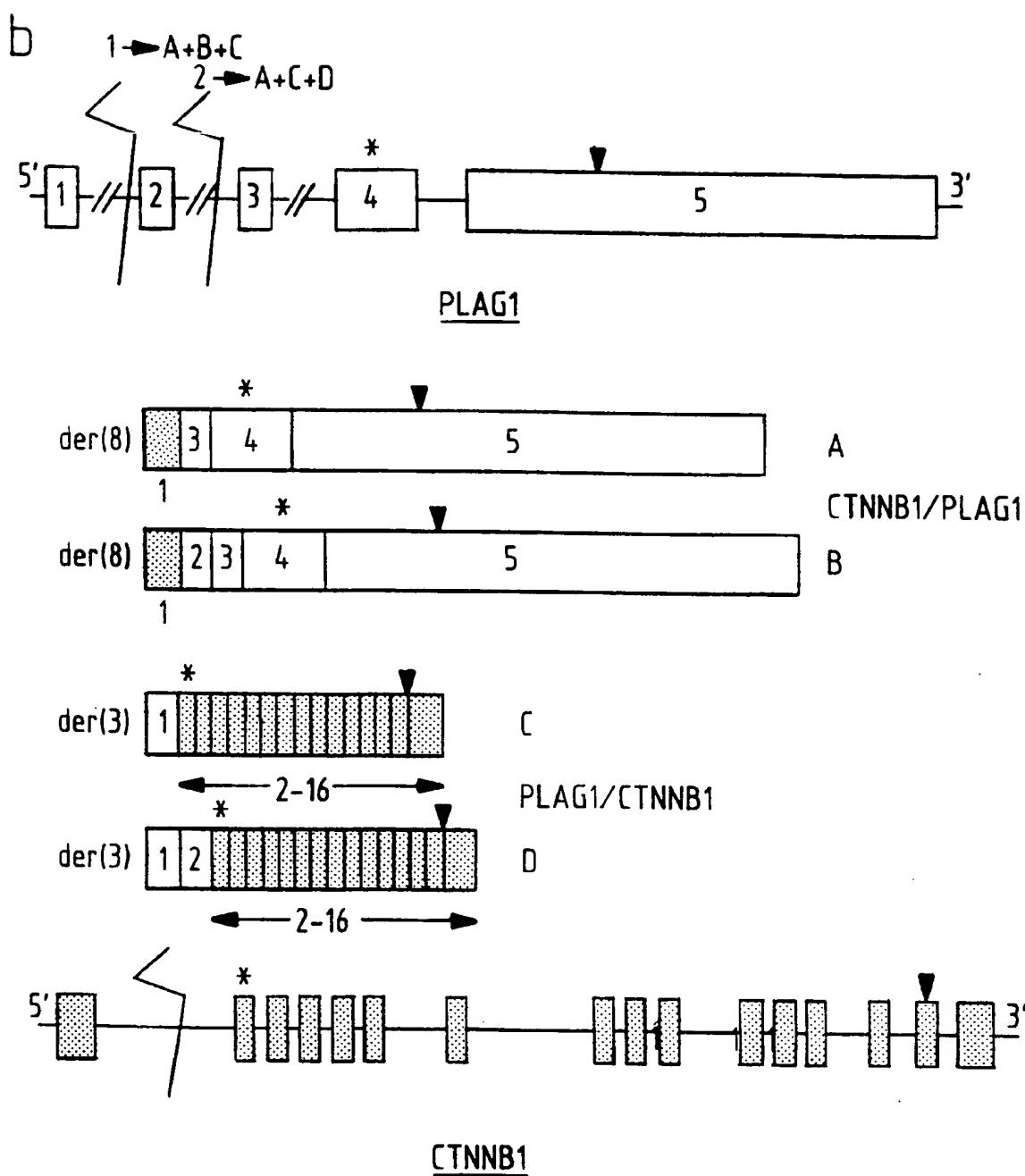
1 2 3 4 5 6 7 8



↑↑
C D

FIG. 6A

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FIG. 6B

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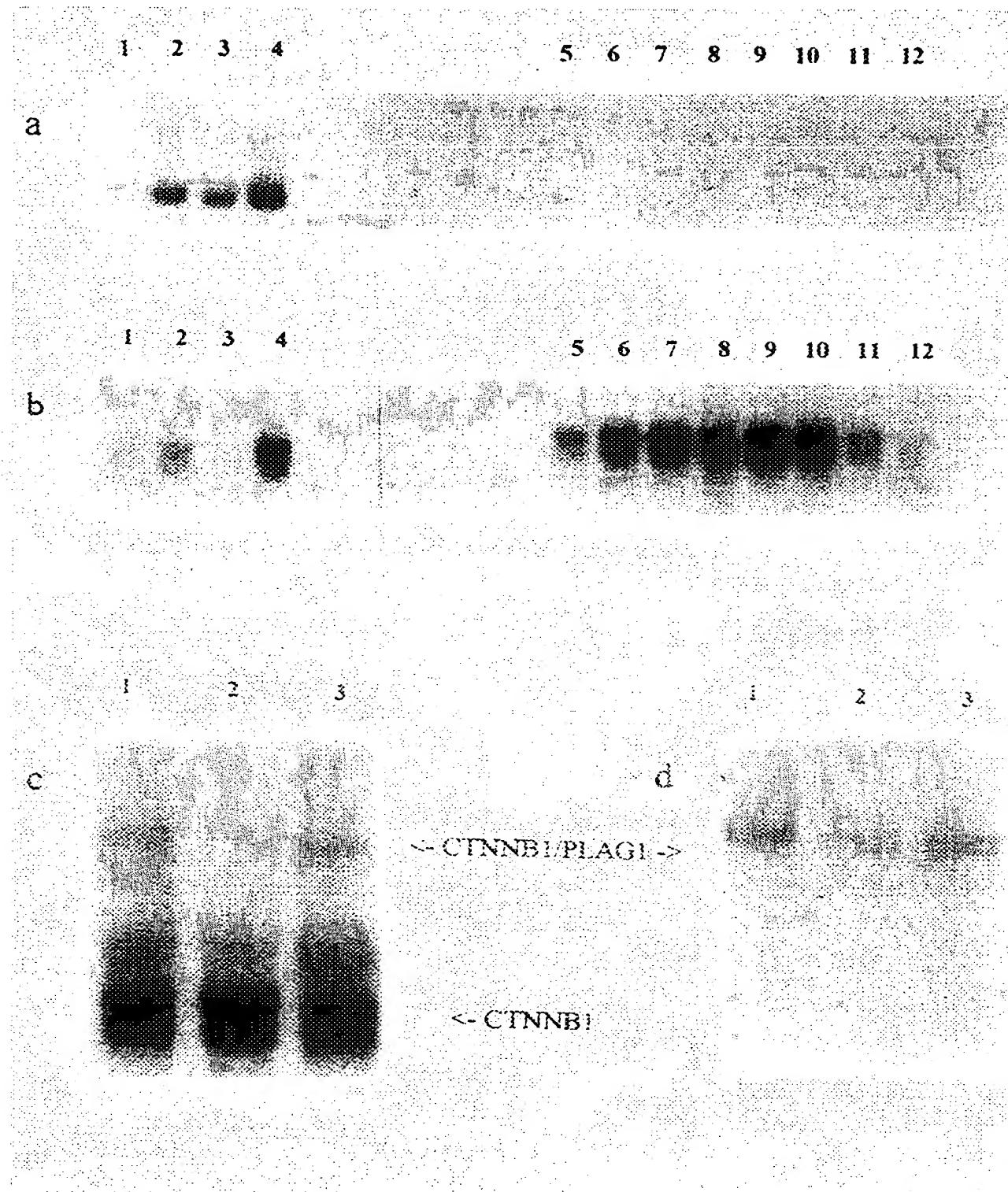


FIG. 7

FIG. 8A

PLAG2 cDNA and Open Reading Frame (underlined)

AGGCTCAAGATAAAGACCTTAACCTTGTGTTCTCCCTTCTAGGTATTGCATAGGAATCAGAGA
 GTTAATCTTGTCTCTTCACAGGTTGAATCTTCAGACAACCTTCTGGAGGACTCGGTCCCTGCGAGCA
 GATGTTCCCTGTCACTCACTAGGCAATATGGCTACCCATTCTCCCAGAAATCTCACCCAGTGTCACTGTGAGA
 AGACGTTCAACCCGGAAAGACCAACCTGAAAGACCAACCTCCAGACCCACGACCCAAACAAATGGCCTTGGTGTG
 AGGAGTGTGGAAAGAAGTACAACACCATTGCTGGGCTATAAGAGGCCACCTGGCCCTCCATGGGGCCAGCAGTGGG
 ACCTCACCTGTTGGGTCTGTGCCCTGGAGCTAGGGAGCTAGGGAGCTAGGGAGCTAGGGAGCTAGGGAGCTAGGG
 AAGAGAAAGCCCTAGGGAAACCAAGGAAAGGACCCAGTGGGACCAACTGTCAGGATGCTGAAGATGCTTCTACACCCGA
 AGGATGTGGGACCCACCTGGTCCACACAGGATGCAAGGACTTCCCTGTGCCAGGTCTGTGCCAGAGATTG
 GGGCAAGGTTCACCTCACCGGGCATACCAAGAACCCACCTCACAGGAGCTGATGAAAGAGGAGCTTGCAGACCG
 GAGACCTCTGAGCACCTCCACACCATTCTCGCCTTCAGGAAACGGGCTTGCAGTAAAGTCAAGGACTTCCCTGCCT
 TAGGAGCTTCTGCCCCAGAACGGCTATGCCAGCCGGCTGCCAGAGTCCCTGGCTCCACCCCTCGGTATCCCCGGCTCTC
 AACAGGGCCCCAGCCTATGCCAGCCGGCTGCCAGAGTCCCTGGCTCCACCCCTCGGTATCCCCGGCTCTC
 CTCGGCCACCCCTCCCCAATCACAAAGTACAACACCATTCACTTCATACTCCCCACTTGCACGGCTGCCCTCTCA
 AAGCAGATACTAAAGGTTTTGCAATATCAGTTGTTGAGGACTTGCCTCTGCCTCTGCCTCTGCCTCTGCCTCTGC
 AGCTCAACCCAGGTTTGATCTGGCTAAAGGAAATCTGGCTGCTGACCTCTGGCCCTGGGAAATCTGGCCAGCAGT
 CTGTAACCTAACATAACCTGGCTCTCTGGCCATGGGCACTGTGAGCCTGGCCAGGAGCTGCCCTGGCTCTGC
 CCCAAATAACCTTTGGGAATAGCAACTCTGGCTCTCTGGCCATGGGCACTGTGAGCCTGGCCAGGAGCTGCCCTGG
 GGCAGCAGGCCAAGAACCCCACTGGCTCTGGCCACTCTGGCTCTGGCCATGGGCACTGTGAGCCTGGCCAGGAGCT
 ATGTTGTTCTCAGCTGGCCACTCTGGCTCTGGCCATCTGGCTCTGGCCATGGGCACTGTGAGCCTGGCCAGGAGCT
 TGTATTTTCCGTATTCTGGAAGATGTTAAAGAACCTTAAATGTCAGTTACAATAATGAGAAAGATTGGAA
 AACAGAGACTGGACTATGGCTTATTCACTGATGACTGGCTTGAGATGATAAGAGAAATTGGCCACAGTGGTT
 GTGCCAATCTGTCCTGAGTTCTAGCTTGTACCCATTAAATGAAACGGCTGTTCTGTAATCCAAACTGCAAAAT
 ATTGTCATAACCAACATCCAAAATGACGGCTGCTATAATAAGTGTGTTGTCATATGGAAATTAAATGTA
 CCATGGATCCATAATGTTAAACTAAACTTATGGCTGAGCTTCACTTCAGGTTCACTGCTAGTAAGGAACTATGG
 TTCTCCAAATCTGGGAAGATTTCAAATAACCTTTATATGATAACCTTGTACTTACCCATAGCCAACTGTAGTT
 TCTTTCAGGGATTCTCTCAGGGTTGGATGTTAGTTACTTACCCATAGCCAACTGTAGTT
 CATATACATTCTCTGGGAGCAATAGGTTCTCCATTACAGAACCTTAAATGAGCATTAAATGTA
 ACAAGATGCTGCAATGTGAGTTACCTTCATTATGTTAAAGGGAAATTAAACCTACTACCTCAAGGTCTAG
 AGAAAAAAATCTACGGGTTAACTGTTAAAGGGAAATTAAACCTACTACCTCAAGGTCTAG
 CTGACTTTAAATTGATTAATTCTCCCTTGGCTCTGGCTTAAAGGTTAACTGTTAAAGGTTCTTAAAGGTT
 TATTAACAGAAATTTCACCAATAAAAGTGTAAATTCTAGCTTAAAGGTTAACTGTTAAAGGTTCTTAAAGGTT
 GTTTACAATGTAACCTAAATCTCTGTAATTGGTATCTTAAAGGTTCTTAAAGGTTAACTGTTAAACCT
 TTAAAGGCTCCATTGTCCTTATGTTAGGGCTTTCCGTAACATATCTTACATATAAACCTTAAACCTTCAAA
 TCTTGCAAATT

PLAG2 protein

MATHSPQKSHQCAHCEKTFNRKDHLKNHLQTHDPNKMAFGCEEGKKYNTMULGYKRHLALHAASSGDLTCGVCAL
ELGSTEVLDHLKAHAEEKPPSGTKEKKHQCDHCERCFYTRKDVRRLVVTGCKDQLCQFCQAQRFGRKVHLTRH
TKKTHSQELMKESLQTDLILSTFHTISPSFQLKAALPFPFLGASAQNGLASSLPAEVHSLSLSPPEQAAQPMQP
LPESLASLHPSVSPGSPPPLPNHKYNTSTSYSPLASLPLKADTKGFCNISLFFEDLPLQEPPQKLNPQFDLA
KGNAGKVNLPKELPADAVNLTIAPASLDLSPLJGFWLQPLPPATQNTFGNSTIALLGPGESELPHRLSCLGQQQQEPPL
AMGTVSLGQOLPLPPIPHVFSAGTGSAILPHFHHAFR.

FIG. 8B

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FIG. 9 Nucleotide sequence of cDNA of CTNNB1 (β -catenin)

1 aaggcctcg gtctgttgc cagcgttgg cccggcccg ggaggggagg gcgggggg
 61 gccccccg ttcagtcgg gccgagccgc caccgcagg
 121 cggactccg cggggagg agccctttcc ccttgaggta ttgtgatgt
 181 accataaca atccagggtg gacaatggct actcaaggctt atttgtatgg
 241 gttggacatg gccaatggaa cagacagaaa agcggctgtt agtcaactggc
 301 ttacctggac tcttggatcc attctggatc cactaccaca gctccctctc tgagggtta
 361 aggcaatccctt gaggaaaggat atgtggatcc cttccaaatgtc ctgtatgtat
 421 attttcttag tccttactc aagaacaatgtt agctgtatattt gatggacatgt
 481 tcgactcttag agggtacatgg ctgctatgtt ccctgagaca tttagatgggg
 541 cccatctaca cagtttgcatgg ctgctcatcc cactaaatgtc cagcgttgg ctgaaccatc

 601 acagatgctg aaacatgcag ttgttaaactt gattaactat caagatgtat
 661 cacacgttgc atccctgttgc tgacaaaact gcttaatgtac gaggaccagg
 721 taaggcttgc gttatggtcc atcagcttcc ataaaaggaa gcttcaggac acgctatcat
 781 gcggttctccat cagatgggtt ctgcttattgt acgttaccatg cagaataacaa atgtatgt
 841 aacagctcgat tggatcccgctg ggacccttgc taaccccttcc cttccatcgatgg
 901 ggcctatctt aagtctggat gcatctctgc cttccatcgatgg
 961 ttctgttgc tttatggca ttacaactctt ccacaacccat
 1021 taaaatggca gtgcgttttag ctgggtggct gcagaaaaatg
 1081 aaatgttaaa ttcttggcttta ttacgacaga ctggcccttcaa
 1141 agaaaaggcaag ctcatcatac tggcttagtgg

 1201 gacctataact tacgaaaaaac tactgtggac
 1261 ctgctcttagt aataagccgg ctattgttgc
 1321 cctgacagat ccaagtcac
 1381 agatgtgtca actaaacagg aaggatggaa
 1441 gggttcagat gatataaaatg
 1501 caataattat aagaacaaga
 1561 tactgtccctt cgggtggat
 1621 tcatctgtac
 1681 tggactacttaca
 1741 tactgttggaa ttgatttcgaa

 1201 gacaaggcaga tactgtggac
 1261 ctgttgc
 1321 gaaactgttca
 1381 tggactcttc
 1441 gggactctt
 1501 ttcaatcttca
 1561 ttcaatcttca
 1621 acgttgcac
 1681 aatgcgttca
 1741 cactggccctc

 1201 tgctatctgt
 1261 taggacttca
 1321 ggaatcttc
 1381 ttcaatctt
 1441 acctacttgc
 1501 ctcttgc
 1561 cctggccatct
 1621 gtgcttcc
 1681 gccttcaact
 1741 tgataaaaggc

 1201 gtgtgtgg
 1261 atgcggcagg
 1321 tggactcttc
 1381 ggtatgggtt
 1441 ttcaatcttca
 1501 ccaatgtgg
 1561 ttcaatcttca
 1621 acgttgcac
 1681 aatgcgttca
 1741 cactggccctc

 1201 ttgttccctt
 1261 atcttgcac
 1321 aatcatgtgc
 1381 ttgttccctt
 1441 cactggccctc
 1501 tgataaaaggc
 1561 ttgttccctt
 1621 aatgcgttca
 1681 cactggccctc
 1741 ttgttccctt

FIG. 9 (continued)

FIG. 10

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STSs used to generate the 300 kb cosmid contig mapping at chromosome 8q12 and encompassing PLAG1

STS CH129

GAATTCTAAAACCATTATAAATCATACTGAATCCCAGAACAAATATTTAAACAACCTAA
AAAAAGAACAAAATAAGCAAAACATTAAAGAGTGTAGATTCTTGAAATTAAAGG
ACATACTTACCCCTGTAGT

STS CH280

GAATTCTGCACCGGTTTTCTTATCAGTGTGGGCTGATGTTCCATTAACGTGGTGTAAAT
TTGAGTATAGTCAGTCACTGACTGATTCTAGATATTTCAAGAGGTCAAGACTTTCTAAGACCT
TTATATGTGGTTGAATTCTGTTCTGGTTCACAGAAGGTATATTAGCAAAGCATTGG
TGTTGAAGCTTGGTCTGTGATCTAGT

STS CH33

GAATTCGTTTTATTGACAAGCACATGAAGCCTTATCAGACGGAGGCCTCAATCCTTGCG
TGGGGTTATAAGCAGGTAGCGCTAGACCTCCATTCTACATAAGCTGATGGCACGGTAA
TAGCTGGGGTTTCTCACAAAGTCAAAGACAATTGCTGTGTTCAAGCGTGTGAAACAGTT
WAAWACGTTGAGGTCTCTCTGCTCATAGGCCATCTGGCTCAGACATTCTACAGMCA

STS EM156

TCTGAGCAACAAGAGCGAAACTCCATCTCAAAATATATATATATAGGTAATTGTTGTCAT
TAATATTAATGTAGTAGCAGCAGCAACAGTCATGGTAGCAATATTGCTCTATTGGGAGGCA
ACTTATAATTATTAACTGTGGAATATCTTGGAAAATGTTTNGCAGAMGTTATGTTCCA
TTCCTGACTGGMGCTCATTATAAATACCCATCTCTGAATAGCGCAAGGACTTTGAAA
AGTGTCTGAGTAAAC

STS EM195

ACAATCAATTAGAAAGTAATCATTCACTACCCAAACTGAAACCTGTACCTGTTAGCA
CTCACTCCCTTTCTTCACTTTACTTTTATTATTTGAGAGAGACTTGCTCTATC
GCCCNNGCNVCAGTGCAGTGGCACAACTCAACTGCAACCTCTGCCAGGGTCAA
GTGATTCTTGTGCCTCAGAGTCCAAGTACCTGGGATTACAGGCATAAGCCACCGCCTGG
CTAAATTGTTGTATTTCAGTAGTGACGGGTTTCACCATGTTGCCAGGCTGTCTCAAAC
CTGACCTCAGGTAACTCACCCTCTCAGCCTCCAGAGTTCTGGGATTACAGGCCTGACACC
GTGCCTGGCTCATTATTAGAGATGAGATCTCACTCTKWTGCCAGGCTTCAGTGC
ATTGGCGTCATGATGGCTCACTGCAGGCTTCAGCTCCTGGCCTCAAaGCATCCTCCGCCTCA

STS EM208

CTAGGCAGAGCAAGACTCTGTCARGAAAAAAaVRAAAAAAATTACCAAAAC
TGACTACAGAAAAAVHGVARGGTTGAATAGCCTTACATTGGVAAATAATTAAATT
TAAAGATATTAAATTAAAVVTACTCTAGGCCATAAGGCTTCACAGGTTAATTGTATTAA
TATTAAAGGAAAAATAACCAATTCTATTGATAGCTTCAAGAAAATAGAGGCGTATCCA
TTTTCTAACTCATTAAAGAAATAGCATCATTCTAAATATCAAAGCAACAAAGMCATTGC
AAAGAAGAAGGGAGAAGAAGGAAGAGGAGAGGAGGAGAAAGGGAGCAGGAGATGGAGAAGA
AGGAAGCCAGGTACAGTGCATATTCTCATGAACATAAACACAATTAAAGTATTAM
CAGGCTGGCTTGGCTCTCACCCGAATCCAGCMCTTGGAAGGCCAAGGCAGGTTGG
GHCACAAGGTCAAGGGTTCGAG

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FIG. 10 (continued)**STS EM216**

TACTRACTGCTGTGCAGTTBTC_cTGCAGTCAGTCCAGAGGTCAATTCTAACGTTGCACTAT
 GGGKCTATTAAATAGGTTCTAAAGAACAAACATATCTCTTAbAGTTACTCAGAGGGTAC
 ACAATGATGATGTACACAAATTAAATTACCTATTAAAGACTGAAATCCAGCAATGCATAGKGTG
 TGGACTTTACGCACATCCAGAAAAAGTTCTAGCACAAATTGTTTHGTMYATATATTTCAG
 AAGCCATAGAAACACTATTAAAGCCCTCCCTAACACTAGGGATGCAAAaTCAATAT

STS EM317

GACCAACAAAGGCACACAAAGATTGGTTGCTTCTGAAGAATCTAAAATGGCATTGGGTAT
 AGGAGTTGGGGAGCAAGTTGTATAGGCACCTACACTTAAGATAATTGTCAATTATACAAA
 TAATTTTAAAGTTAAGCCCCTTCTGACATGACACGTCCATGGGTCTTCACCCTY_{TtK}
 KTCTCCTSCAGAGCTCCAGTCTGCCYYTTT_KSCTCTGAGCTCCAAAAMCAGTGAWTCCCC
 TGAAGTTACCTAGMCCMCCATACAGTTGTGACTCCCTAWM_cGGGGTACCyTCCCATGY
 CTGGCTAATAYTGABTYTTGTDACCCTGGTTCTGTGTTACTACATTGTTARTGGAAT
 TWAT_WAArGGGAAGCCTATCAA

STS EM416

GAGCAACTGA_aCDNAGATTGGGTGAGCTAAGATGTGGGCTGCACAGGTGAGGCTGGAGAGGT
 GGGGAGTGCCTCCAGTCGGGGAGAAGAAGAAAAGGGCAGACTAGGGTAGAAATGCTTATW
 ACT_cCTGTGACTGGAGCTGATGGTGTCTTAAGGAAAGTGGTGGGAAGGGAGGVCTGCAGAAA
 GGCAAGGCTGGAGTCGACTGAAGGCTGGAGAGGCCACTGCTTAAACAAGTGTAMCTGGAGATG
 GAAGGGGCTGCAGGACAGGTCACTCAGCCAGTKGTGARGCAATCTCACC

STS EM443

TTGATATTGTTCTAACTCCACATTAACATTGACAAATACTCTAAATTGTAGCTACCATCT
 GTTACCGTAGCTAGCAGGTACCCCTAACAGCAATGGGTCAAGCTTTGAGTAGCGTTCAACCCT
 GTTACCTCGAGTACGGTGTGGTCAAGGAAAGTGGGAGACGAGATGGAGAGAAAGAAACAGAACGAGC
 ATTCCATTGTTGCTCACAGTCCCAGGGCAAACACAGCACAGCCTACAGGACCATG
 AAGGGGAGCACTGGGTCACTCATGAAGCAGGGAGGTGGGCCAGTGGTGGGGgCCTTAT
 GTGTTTCCTCAGGAAGGAATGGCAAGGCAGGGTAAGCATGTTCAAGGACTGGTTAATTGAA
 ATAACCTCAGGGGGgCTCTAGGGCCTG_gRGGCTGCCCTGGTTCTGGTACCY_gGSCCTG

STS EM46

ATATCAATCTGGGTCTATGTATGTTTGCTTTCCCCAGTGTCCAGGCATGATGCTAAG
 GATATAGGGATGATGAAATATATGCTTGCTGAATATGGAAATAAGAATTATTTATGATCA
 GAHTTTTTTTTGAGATGGAGTCTCGCTCTGTCACnMaGGCTVGTGCAAGTGGCATGAT
 CTCAGCTCACWGCAACCTCTGVCTCCTGGTTCAAGTGGATT

STS EM47

GTAGAGACACACTAGGCATGCACAGACCAAGTGCAGAATGAACAAATTTGTTACATGTGTAG
 TTCTTATGGTTACAAAACCTCTCCAGCCATTATCTTCTTCAAGCCTTATAAAAGACAGAG
 CATATTATTATCCTCATTACCTWHTCTAGTAAGGCATTCTTCTTACTAGA
 GATATAAGGCTTAGGAAAAAGTGAATAACTACGATAAAATGAATACTAGGAAAAGACATCACA
 ATCACAAATTATTAATCAGAAAACAGDTTTAAGAATAAAATWTTCAAWAARgAAA

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FIG. 10 (continued)

STS END2

TAATTTTACTACACTACGGAATTCTGTGCAGTGAGATCAAAGAGCTGTGTATGCCCATATAATGTGA
TTTACAGCCATTTGTAAAAACTGTAAAATACCTTAATATTCAATTGGCTTAAGGTACAT
TGAGGACTTCTGGTTGAAAATTACAGAGTGGTGAAGATTG

Known STSs

PENK

D8S285

MOS

STSs part of PLAG1

EM265

KK64

KK63/EM209

KK55/CH283

EM224

EM387

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<p style="text-align: center;">A3</p>		<p>(43) International Publication Date: 26 February 1998 (26.02.98)</p>
<p>(21) International Application Number: PCT/EP97/04759</p> <p>(22) International Filing Date: 22 August 1997 (22.08.97)</p> <p>(30) Priority Data: 96202339.6 22 August 1996 (22.08.96) EP (34) Countries for which the regional or international application was filed: AT et al. 97200130.9 17 January 1997 (17.01.97) EP (34) Countries for which the regional or international application was filed: AT et al.</p> <p>(71) Applicants (for all designated States except US): K.U. LEUVEN RESEARCH & DEVELOPMENT [BE/BE]; Groot Begijnhof, Benedenstraat 59, B-3000 Leuven (BE). HOLD-INGBOLAGET VID GÖTEBORGS UNIVERSITET AB [SE/SE]; Medicinaregatan 8, S-413 90 Göteborg (SE).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (for US only): VAN DE VEN, Willem, Jan, Marie [NL/BE]; Lei 8A bus 42, B-3000 Leuven (BE). STENMAN, Karl, Göran, David [SE/SE]; Fältskärsgatan 7, S-426 76 Västra Frölunda (SE). KAS, Koen, Pieter [BE/BE]; Kasteelrei 115, B-2110 Wijbegem (BE). VOZ, Marianne, Léontine [BE/BE]; Rue Marcel Hicter 14, B-4300 Waremme (BE).</p>		
<p>(74) Agent: VAN SOMEREN, Petronella, Francisca, Hendrika, Maria; Arnold & Siedsma, Sweelinckplein 1, NL-2517 GK The Hague (NL).</p> <p>(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>With international search report.</i> <i>Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i></p> <p>(88) Date of publication of the international search report: 27 August 1998 (27.08.98)</p>		
<p>(54) Title: PLAG GENE FAMILY AND TUMORIGENESIS</p> <p>(57) Abstract</p> <p>The present invention relates to a novel gene family identified as T-genes and being implicated in tumorigenesis having the nucleotide sequence of any one of the strands of any one of the members of the <u>PLAG</u> and <u>CTNNB1</u> gene families. The genes and their derivatives may be used in various diagnostic and therapeutic applications.</p>		

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C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category ^a	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	N.K. SPURR ET AL.: "Report of the second international workshop on human chromosome 8 mapping 1994" CYTOGENETICS AND CELL GENETICS, vol. 68, no. 3-4, 1995, BASEL CH, pages 148-155, XP002036781 * the whole document, esp. p153, column 2 *	1-4
A	--- WO 89 09777 A (ARCH DEVELOPMENT CORPORATION) 19 October 1989 see the whole document	1
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8 June 1998	13-07- 1998
Name and mailing address of the ISA European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Authorized officer De Kok, A

INTERNATIONAL SEARCH REPORT

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PCT/EP 97/04759

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	A. ABDOLLAHI ET AL.: "Identification of a gene which shows decreased expression in malignantly transformed rat ovarian surface epithelial cells" PROCEEDINGS OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH ANNUAL MEETING, vol. 37, no. 8, 20 April 1996, WASHINGTON US, page 242 XP002036823 * abstract nr. 1654 ---	1,4
P,X	E. ROIJER ET AL.: "Identification of a yeast artificial chromosome spanning the 8q12 translocation breakpoint in pleomorphic adenomas with t(3;8)(p21;q12)" GENES CHROMOSOMES AND CANCER, vol. 17, no. 3, November 1996, NEW YORK US, pages 166-171, XP002036782 cited in the application see the whole document ---	1-4,6-23
P,X	EMBL DATABASE, Heidelberg, DE, Acc.nr. U72621, 22 October 1996, A. Abdollahi et al., "Identification of a gene containing zinc-finger motifs based on lost expression in malignantly transformed rat ovarian surface epithelial cells". XP002036784 see abstract ---	1,4
P,X	A. ABDOLLAHI ET AL.: "Identification of a zinc-finger gene at 6q25: a chromosomal region implicated in development of many solid tumors" ONCOGENE, vol. 14, no. 16, 24 April 1997, OXFORD GB, pages 1973-1979, XP002036783 see the whole document ---	1,4
X	F NOLLET ET AL: "Genomic organization of the human beta-catenin gene (CTNNB1)" GENOMICS, vol. 32, no. 3, March 1996, NEW YORK US, pages 413-424, XP002047790 cited in the application see the whole document ---	1,5-25
	-/-	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	J. HUELSKEN ET AL.: "E-cadherin and APC compete for the interaction with beta-catenin and the cytoskeleton" JOURNAL OF CELL BIOLOGY, vol. 127, 1994, NEW YORK US, pages 2061-2069, XP002047791 see the whole document ---	1,5-25
X	C KRAUS ET AL.: "Localization of the human beta-catenin gene (CTNNB1) to 3p21: A region implicated in tumor development" GENOMICS, vol. 23, no. 1, 1994, NEW YORK US, pages 272-274, XP002047792 cited in the application see the whole document ---	1,5-25
X	J. VAN HENGEL ET AL.: "Assignment of the human beta-catenin gene (CTNNB1) to 3p22-p21.3 by fluorescence in situ hybridization" CYTOGENETICS AND CELL GENETICS, vol. 70, no. 1-2, 1995, BASEL CH, pages 68-70, XP002047793 see the whole document ---	1,5-25
A	RABBITS T H: "CHROMOSOMAL TRANSLOCATIONS IN HUMAN CANCER" NATURE, vol. 372, 10 November 1994, LONDON GB, pages 143-149, XP002056672 see the whole document ---	1
A	WEINBERG R A: "TUMOR SUPPRESSOR GENES" SCIENCE, vol. 254, no. 5035, 22 November 1991, LANCASTER, PA US, pages 1138-1146, XP002056673 see the whole document -----	1

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP 97/04759

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. Claims Nos.: because they relate to subject matter not required to be searched by this Authority, namely:

2. Claims Nos.: 6-23 partially because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:
claims 6-23 could be searched only incompletely because they lack technical disclosure (Art. 6 PCT and PCT Search Guidelines, Chapter III, 3.7)

3. Claims Nos.: because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

The additional search fees were accompanied by the applicant's protest.

No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/ EP 97/04759

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims : 1 (partially), 2-4 (completely) and 6-25 (partially)

Isolated gene implicated in tumorigenesis, having the nucleotide sequence of any one of the members of the PLAG subfamily of zinc finger proteins

2. Claims : 1 (partially), 5 (completely) and 6-25 (partially)

Isolated gene implicated in tumorigenesis, having the nucleotide sequence of the CTNNB1 gene.

INTERNATIONAL SEARCH REPORT

Information on patent family members

Interr. nat Application No

PCT/EP 97/04759

Patent document cited in search report		Publication date	Patent family member(s)	Publication date
WO 8909777	A	19-10-1989	US 5206152 A	27-04-1993
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